TEXTBOOK OF CLINICAL PATHOLOGY

A TEXTBOOK

of

CLINICAL PATHOLOGY

Edited by

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Emory University, Ga.

AND

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SECOND EDITION

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PREFACE TO SECOND EDITION

Although the first edition of this book appeared as late as 1938, it has become necessary to subject it to a thorough revision. This has been brought about by the warm reception that was accorded the first edition for which we are extremely grateful; and secondly, by the fact that the field of clinical pathology rapidly changes since new procedures are being introduced constantly. Furthermore, certain laboratory procedures of importance were inadvertently omitted.

As in the first edition, this book is designed primarily for accurate and comprehensive teaching of this important subject to students of medicine and the interpretation of laboratory findings for the practitioner of medicine. Therefore emphasis is placed upon the interpretation of results. Because of the extensive revision, it has been necessary to completely reset the entire book and the changes are mainly the addition of new material, particularly new procedures that have been introduced in the last two years. Thus, there is an entire chapter on the determination of vitamins and hormones by Dr. Emmerich von Haam of Ohio State University. The chapter on serological procedures in the diagnosis of syphilis has been completely rewritten and enlarged by Dr. I. Davidsohn of Rush Medical School, Chicago, Ill.

In the section on clinical chemistry new and valuable laboratory procedures have been incorporated, including the determination of amylase and phosphatase in the blood and sulfanilamide, sulfapyridine and sulfathiazol in blood and urine. In the chapter on liver function tests will be found the phenoltetraiodophthalein (Iso-iodeikon) test.

Throughout the entire volume, our associate, Dr. Elizabeth Gambrell, has completely revised all material pertaining to bacteriological procedures and this will be found interspersed in various sections throughout the book. This includes new procedures for anaerobic cultivation of bacteria, the preparation of autogenous vaccines, a new section on the preparation of valuable and widely used culture media, particularly ones more recently introduced for isolation of organisms of the colon-typhoid-dysentery group. The section on blood cultures has been rewritten including methods for arterial puncture.

In the field of hematology new material is incorporated on the examination of the bone marrow, the absorption tests for infectious mononucleosis as well as two new color plates on spectroscopic examina-

tion of blood and urine for hemoglobin and its derivatives. Also there is a new section on the role of vitamin K and the determination of prothrombin in the blood.

Under miscellaneous laboratory tests will be found methods for examination of seminal fluid and the quantitative Friedman test. There is a complete section on skin tests including those that are employed in Brucellosis, the skin tests for trichinae and echinococcus, tuberculin test, and the Dick and Schick tests. The precipitin tests for animal parasites is described as well as the digestion method for trichinae. A chapter of unusual value by Dr. von Haam is that on the diagnosis of venereal lesions, including all of the widely approved methods for the diagnosis of the chancre, chancroid, granuloma inguinale, lymphopathia venereum, venereal fuso-spirochetosis, and the gonococcal lesions.

The illustrations of this edition have also undergone a thorough revision being increased in number from 240 to 257 which includes 34 full page plates, 23 of which are in color. Although the total number of illustrations have been increased by only 17, actually there have been added 47 new figures.

In the preparation of material for this edition we express our indebtedness to those who have been of invaluable aid. Among these are Dr. Evangeline Papageorge of the Department of Bio-Chemistry in the Emory University School of Medicine who prepared all of the solutions of hemoglobin and its derivatives from which the color plates of absorption spectra were drawn. We are also indebted to Miss Blanche Lockard, assistant in Bacteriology and Pathology at the Emory University School of Medicine, for numerous suggestions and criticism; to Miss Emily Harris, graduate student in the Department of Pathology and Mrs. Herma Gibson, secretary in the department, for untiring efforts in the preparation of the manuscript. The new illustrations for the most part have been prepared by Miss Frances Baker. We would be remiss in our duty if we failed to express appreciation to our colleagues who are contributors to this volume for their enthusiastic cooperation in this work.

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Roy R. Kracke
Francis P. Parker

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Emory University, Ga.

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CHAPTER 1

PREPARATION AND STAINING OF BLOOD FILMS: VENEPUNCTURE
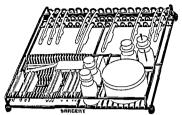
By A. J. Miller

PREPARATION OF THE FILM
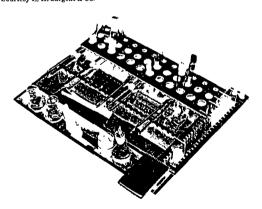
General Considerations. Blood may be prepared for microscopic examination in several ways. It may be examined in its fresh state, unaltered by staining, by placing a small drop on a plain glass slide, placing on a cover glass and then sealing the edges with petrolatum to prevent evaporation. In this way the red cells may be observed in their natural state, certain parasites may be detected, particularly those that are motile, and motility of white cells may be studied, especially if the preparation is kept at body temperature during the examination. Furthermore, a drop of blood may be mixed with neutral dyes such as neutral red and Janus green, and studied in a similar way. This is known as intravital or supravital staining. Certain cells may be identified by the amount and type of dye taken up and by its distribution in the cytoplasm of the cell. Also some idea of the functional capacity of white cells may be obtained by such methods. When a drop of blood is mixed with a solution of cresyl blue, the reticulum of young red cells becomes stained thus identifying the cell as a reticulocyte.

For routine studies, however, blood is nearly always examined by preparing a thin film on a glass slide, and this stained with one of the Romanowsky stains. In this way the cells are spread in a thin film over a large surface and each may be studied individually. The blood film may be examined either stained or unstained. The stained film may be used for the differential leukocyte count, in which the various white cells are studied, classified, and tabulated; to determine morphologic alterations in the erythrocytes; for counting blood platelets, and searching for parasites, particularly those of malaria, trypanosomes and filaria.

Furthermore the dried blood on a film may be dissolved in water and used for some of the agglutination tests. Thus the method of smearing and staining blood has come to be one of the most important procedures in laboratory diagnosis. Blood films should be made with careful attention to details of technic, since no examination is quite so unsatisfactory as that of the poorly prepared blood film. A good blood

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FIG. 1. A suitable tray and carrier for routine hematologic work in a hospital. Courtesy E. H. Sargent & Co.

placed back to back; clean cotton beneath small pad on corner. (Kracke & Garver)

film should cover the middle third of the slide, with a gradual change from a thick to a thin portion, and should contain an even distribution of leukocytes. To make such a film no more than one-fourth of a drop of blood should be used.

Obtaining the Blood. Since only a small amount of blood is required for blood films, this usually is obtained from puncture of the side or ball of the finger, the lobe of the ear, the heel or toe of an infant, or if vene-
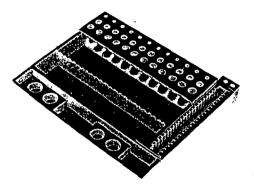
Fig 3. Same tray as shown before   It can be constructed by any good carpenter with variations to meet the requirements of any particular laboratory. Designed by Clara M. Becton, St Johns' Hospital, Tulsa, Okla. Courtesy J. B. Lippincott Company, from Diseases of the Blood, by Kracke and Garver, 1937.

puncture is being done for other purposes an adequate amount of blood may be expressed from the needle in a small drop on the end of a glass side. There is little or no difference in the findings of capillary and venous blood. Puncture of the ear is said to be less painful but the finger is more widely used since it is more accessible and more easily manipulated.

The materials necessary for finger puncture include clean glass slides,

a suitable instrument for puncture, alcohol, and cotton.   Improperly cleaned slides, particularly greasy ones, cause uneven spreading of blood. The resulting irregular bare areas give the film a moth-eaten appearance. Slides should be washed first in warm soapy water, then in 95 per cent ethyl alcohol and finally either stored in clean alcohol or dried with a lint-free cloth and kept in slide boxes.   This technic is particularly desirable when making films from extremely anemic persons since such blood usually forms bare retraction areas.   Various instruments have

FIG 5. A satisfactory instrument for finger puncture which consists of a small bottle containing alcohol with a cork stopper through which is placed a slender Bard-Parker knife blade which projects sufficiently to make an incision of the desired depth   One nib of a pen point can be broken off and the remainder projected through a cork in a similar manner.

been used for making the puncture.   In general it should be sharp, free from rust, have a cutting edge, and be kept sterile.   A small lancet that makes a linear incision of from one to three millimeters is satisfactory. Puncture should not be more than two millimeters in depth.   Some instruments have an adjustable guard that allows the point to project only a certain length.   This is good for a novice since it permits a hard stroke with no danger of puncturing too deeply.   Others are spring lancets that work on a similar principle.   The Hagedorn needle is excellent.   A sharp pointed Bard-Parker blade with the tip projecting through a cork is

quite satisfactory. The cork can be kept in the alcohol bottle and the sterility of the point is always assured (See Fig. 5). Also one nib of a sharp pen point may be broken off and the other pushed through a cork in a similar manner. It is not advisable to use round needles or pins
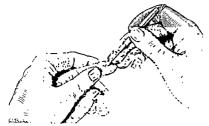
Fig. 6. Showing method of puncturing the side of the middle finger to obtain blood. The ball of the finger can also be used. Note that the operator holds the finger tightly after having stroked it to promote circulation

Fig. 7 Making light pressure around the puncture wound and showing the welling up of a drop of blood. The first drop should be wiped away, the finger surface completely dried so the drop will "stand" instead of flowing over the finger.

for finger puncture since the hole is small and round, often too deep, closes quickly and very little blood will flow from it

The cut should be of such length and depth that blood will not spurt nor drip from it, but should be sufficient to permit several drops to be expressed with little pressure about the wound. If it does not flow

freely there will result admixture with tissue juice with resulting errors in the preparations to be examined.

The finger should first be sterilized well with 70 per cent or 95 per cent ethyl alcohol on a cotton pledget, and then dried before the puncture is made. The finger is held with the left hand and can be stroked or "milked" to promote circulation. If it is cold it should be immersed in hot water in a basin or under the tap and rubbed briskly. Pressure proximal to the puncture site producing congestion will lessen the pain. If sticking the ear, hold the lobe between the finger and thumb of the
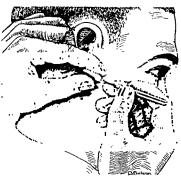
Fig. 8. Showing method of puncturing the lobe of the ear to obtain blood. This is said to be more painless than finger puncture.

left hand, and steady the right hand holding the puncture instrument against the cheek of the patient

After the puncture a drop of blood will well up from the wound. If the skin surface is dry it will "stand" in a large drop and not run over the side of the finger. If the surface is wet it usually flows over the finger of both patient and operator. The first drop is wiped away with a piece of cotton and gentle pressure is exerted proximal to the puncture wound expressing a smaller one for the specimen to be placed on the slide.

Transfer of Blood to Slide. Carefully touch the bottom surface of a clean slide to the drop of blood, leaving a small drop near one end

where the film begins. The slide is then held in the left hand or placed on the table top with the drop of blood uppermost. A second or
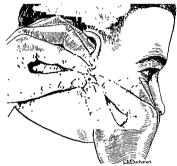
FIG 9. Appearance of drop of blood on lobe of ear
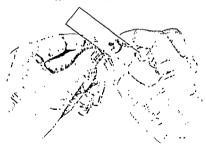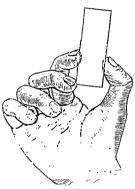
FIG. 10 Placing drop of blood on the bottom surface of a clean glass slide. The drop should be small and placed near the end of the slide.

"spreader" slide the end of which should be smooth, is then grasped between the fingers and thumb of the right hand and its end brought in

contact with the drop of blood. The blood then spreads laterally in the
angle of about thirty degrees that is formed between the two slides
After it has spread across, the spreader slide is pushed forward to the
end of the blood slide pulling the layer of blood behind it. In this way
the blood spreads in a thin film and is dragged behind the edge of the
spreader slide. It is important that the end of the spreader slide back
into the drop so as to pull the blood rather than pushing the blood
with the crushing of cells that would result.
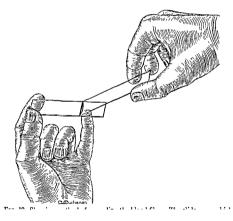
the clean slide that is to be used as the spreader
Note that it is held by the edges between thumb

The spreader slide should be pushed with a steady slow movement.
Unsteady tremors result in waves and ridges in the blood film. The
thickness of the film depends on two factors, the speed of the spreader
slide, and the angle at which it is held. |The greater the angle the thicker
the film and the faster the speed the thicker the film| Since blood
films should be thin, the angle should be small and the rate of spread
should be slow. A proper angle of the slide results in a more even dis-
tribution of leukocytes. A suitable blood film should have a very thin
area at one end and one relatively thick at the other with gradations
between the two. For detailed morphology the thin end is used and in

searching for scarce parasites as in malaria and for leukocytes that are
infrequent as in agranulocytosis, the thick portions are satisfactory.

In searching for malaria parasites thick films can be made, but it is
then necessary to dehemoglobinize the film before or during the staining
process. For details of this see p. 224.

Blood films should be allowed to dry in the air This requires only
a few seconds or a minute Heat should not be used. The smear can

then be labeled with the date and name of patient by writing on the film
with a piece of glass such as the corner of another slide, or with an ordi-
nary pencil in the thick part of the smear. Blood films should be
stained immediately or within a few hours after they are made. Old
smears do not stain well presumably because of the hardening and drying
of the plasma between the cells so that the entire film may appear
discolored. Two or more films should be made from each patient. If

these are not to be stained immediately, they should be turned face downward on the table for protection. One fly, in a few minutes, can eat the blood film from a slide or so damage it that it is unfit for examination.
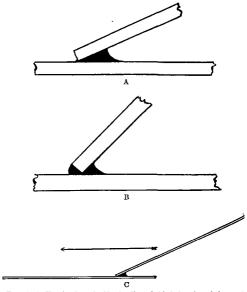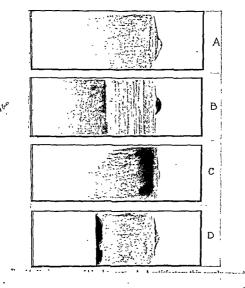
Fig. 13. A Showing how the blood collects behind the edge of the spread slide when the angle between the two slides is small. B. Showing how blood collects in front of the edge of the spreader slide when the angle is too great. C. Showing the direction in which the slide is moved to make the blood film.

In preparing thick films for malaria a large drop of blood is placed the center of the slide and gradually spread to the size of a ten cent piece, using a corner of another slide, a toothpick, or glass rod it

rotary motion for several minutes until the spread is firmly adherent to the slide. This prevents it coming off during the staining process.

Fixation of Blood Films. Blood films should not be fixed with heat, because of resulting distortion of cells. Either methyl or absolute

ethyl alcohol for one or two minutes is an excellent fixing agent. The fixing agent should contain no water. If a stain is used that is contained in methyl alcohol the fixing and staining is then carried out simultane-

ously.   This is the case in Wright's stain and most other Romanowsky stains.   In the Giemsa method it is necessary to first immerse the film in methyl alcohol before staining since this stain is in aqueous solution. In thick smear *staining for malaria parasites it is necessary to first* fix the films before the hemoglobin is removed with water or other agents.

Cover Glass Method for Blood Films.   Some workers prefer blood films on cover glasses rather than glass slides.   *It is said that cover glass films are thinner, give a more even distribution of leukocytes,* cause less distortion of cellular elements, and in general are more satisfactory.   On the other hand, they are more difficult to prepare, more

Fig 15. Showing method of labeling a blood smear by writing on it with an ordinary lead pencil.

easily broken, more trouble to stain and mount, and if not done by one who is expert, the average preparation is not as good as that on a slide.

First place a small drop of blood on the surface of a square cover glass (No. 1, ⅞ inch) which has been carefully cleaned and brushed free from dust and lint.   Then drop another cover glass upon it.   The blood will then spread in a thin film between the two glasses   The drop should be of such size that the film does not quite reach the edges of the glasses. Then at about the time when the blood has reached its maximum spread, the two cover glasses are pulled apart in a plane parallel to the surfaces, holding them by their diagonal corners.   They are then placed blood side up to dry, after which they are stained and mounted with balsam on slides with the blood side either up or down.   If the cover glasses are thick (No. 2) mount with blood side up so the oil immersion objective can be used.

There is also a method for making blood films that utilizes one slide and one cover glass.   The drop of blood is placed on the slide and the cover glass dropped on.   After the film has spread, the glass is pushed the length of the slide.   An old method was to place a drop of blood on a slide, cover it with a cigarette paper, allow the blood to spread, and then drag the paper the length of the slide.

STAINING BLOOD FILMS

General Considerations. Blood films are stained with aniline dyes of both acid and basic types.   According to McClung aniline dyes are classified according to their chemical behavior, as basic and acid.   Basic aniline dyes are those having the staining radical in the position of the base in a salt and therefore, they stain the acid parts of the cell like the chromatin and nucleus.   Acid dyes, on the contrary, are those in which the radical is in the position of the acid in the salt, and they stain the basic parts of the cell such as the granules in eosinophils.   Both types are employed because certain structures in the blood are acid in character and others are basic.   Thus the cellular elements that contain chromatin material such as nuclei take the basic stain and are said to be basophilic in character, yet they are actually acid in reaction.   Those that take the acid dye are said to be acidophilic or oxyphilic, and if eosin is used as the acid dye, are said to be eosinophilic.   These structures, however, are actually basic in reaction.   From these characteristics many of the white cells derive their names.

The best and most widely used dyes are methylene blue which is basic and eosin which is acid.   When the two are mixed in a single solution, it is then referred to as a "Romanowsky" stain because the Russian physician, Romanowsky, originated the method of combining the two dyes.   Also such preparations are called polychrome methylene blue stains, because of the varied color reactions produced in different cells (polychrome: many or different colors).

Of the various stains of this class the best known are those of Wright, Giemsa, Hastings, and Jenner.   There are many others, but all utilize the same principle.   Wright's stain enjoys wide use in this country, but the others are employed mainly in the European countries.   For routine purposes in the study of blood, Wright's stain is simple to use, is an excellent stain, and is satisfactory for practically all purposes.

Staining with Wright's Stain.. The stain can be prepared in the laboratory or purchased from laboratory supply houses, either in the form of a powder to be mixed with the solvent or in the prepared solu-
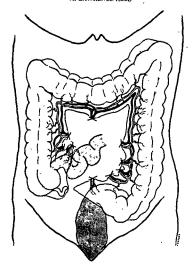
Fig. 7.   Diagram to show the extent of colon removed for ulcerative colitis

raised from the wall of the pelvis and sewn over the reconstructed area so as to render it extraperitoneal, a manœuvre suggested to me by my old chief, Wilfred Trotter.   He never had the opportunity, he told me, to perform it himself, but I have used it in hundreds of cases of colon surgery. Provided the region is drained any leakage that might occur is extraperitoneal.   For drainage I prefer the Babcock stainless steel sumpdrain with continuous gentle suction.

Another method of anastomosis is end-to-end with a tube passed either through the anus and thence through the anastomosis or, as Aylett does, by bringing a knuckle of ileum to the surface to form a temporary safety opening.

A third method is to close both ends and anastomose ileum to rectum side-to-front.

My colleagues and I have now performed total colectomy plus ileo-rectal anastomosis on eighty-one patients (Table VII). A large proportion of these were desperately ill and though the mortality was 9·3 per cent. this is very much lower than if no surgery had been undertaken.

TABLE VII
Ulcerative Colitis
Gordon Hospital 1947-1956

Total cases	..	..	..	.	..	.	567
Number operated	..	.	..	.	.	86
Total colectomy + ileo-rectal anastomosis	.	.	..	81
Total colectomy + removal of rectum	..	.	.	2
Operative mortality	..	..	..	..	.	9·3 per cent.

In two others the rectum had to be included and they are the only two with permanent ileostomy.

In this group of cases, even more if possible than in all colon surgery, the greatest attention must be paid to the avoidance of electrolytic imbalance, and to their difficult and often worrying post-operative care.
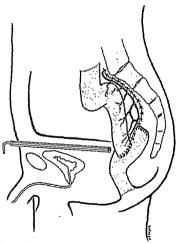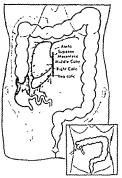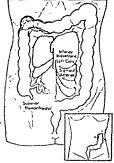
Fig 8   Ileo-rectal anastomosis completed and Babcock sump drain in position.

In cancer of the colon the object of the surgical manœuvre is to ensure the removal of the widest possible area of lymph drainage together with not only the tumour-bearing portion of the bowel but also all of the colon which is supplied by the vessels which have to be ligated. Because the lymph vessels run with and the lymph nodes lie with the arteries, removal of the most extensive lymphatic field entails ligation of the main arteries as close as possible to their points of origin.

Another point in technique worthy of note is the placing of clamps on the bowel at the earliest possible time to prevent cancer cells being disseminated in the lumen and later incorporated with the stitches at the

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Fig 9   Area of resection for cancer of cæcum and ascending colon

Fig 10   Area of resection for extensive growth at hepatic flexure and right side of transverse colon.

anastomosis. If the growth is within reach of the sigmoidoscope it is useful to pack some cotton wool through this instrument up against the growth. If that is impossible, a large tube may be passed well into the rectum, and a thorough washout of the bowel contents carried out immediately before clamps are applied. It would appear that cancer cells are best destroyed in the lumen of the bowel, or for that matter in the fat of the abdominal wall, by a solution of 1-500 perchloride of mercury, as enunciated many years ago by my old chief Ernest Miles.

With regard to the method of anastomosis : with the advent of intestinal antibiotics, end-to-end anastomosis appeared satisfactory, but it does not

avoid the small but highly dangerous mesocolic bare areas. Although end-to-end anastomosis is sometimes used, in the main reliance is placed on a careful four layer invagination of both colon ends followed by a three layer side-to-side anastomosis, utilizing two peritoneal aspects of the bowel leaving no cul-de-sac and avoiding the mesocolic bare areas. When the anastomosis is between small and large intestine it appears best to make it end-to-end. Following a right or a left colectomy the anastomosis usually falls comfortably into the right or left colic gutter respectively, where it may easily be covered by a flap of peritoneum raised from the abdominal wall.

Cancer of the caecum and ascending colon (Fig. 9.) requires division of the right colic and ileocolic arteries at their origin from the superior mesenteric artery and the right branch of the middle colic artery at its origin. The whole of this blood and lymph field is removed together with 20cm. of small intestine, caecum ascending colon and right half of transverse colon. Anastomosis is end-to-side with extraperitonealization and extraperiotoneal drainage.

A growth at the hepatic flexure or right side of transverse colon (Fig. 10) requires a wider excision because the middle colic artery must be ligated at its origin from the superior mesenteric artery and the whole of that blood and lymph area removed, namely the whole colon down to the commencement of the pelvic colon. Anastomosis is end-to-side,

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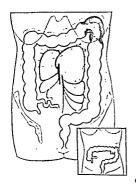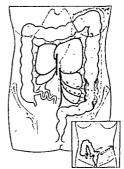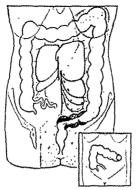
Fig. 11. Area of resection for cancer of splenic flexure

Fig 12   Area of resection for cancer of descending and pelvic colon

(*Reproduced by permission of* Postgraduate Medicine)

Fig. 13. Area of resection for cancer
of pelvi-rectal junction.

ileum to upper-end of pelvic colon with peritoneal flap and extraperitoneal
drain.

For cancer of the transverse colon or splenic flexure (Fig. 11) the middle
colic artery must be divided at its origin from the superior mesenteric
artery and the left colic artery at its origin from the inferior mesenteric
artery. The whole colon supplied by these two vessels is removed to-
gether with the lymphatic fields. Anastomosis is made between the
ascending colon and the pelvic colon.

The procedure for cancer of descending and pelvic colons (Fig. 12),
consists of dividing the inferior mesenteric artery at its orgin from the
aorta. The bowel which it supplies together with the lymphatics running
with it and its branches are removed. Subsequently the transverse colon
is anastomosed to the rectum. Here again extraperitonealization and
continuous suction drainage are indicated.

For malignancy in the pelvic colon including pelvi-rectal junction
(Fig. 13), the ideal area of resection is from the left half of the transverse
colon down to and including the whole rectum, including ligature of the
inferior mesenteric artery at its origin from the aorta. It must be freely
admitted however that many patients are thought to be unable to with-
stand such an extensive resection owing to the greater technical difficulties

as compared with the standard abdomino-perineal excision. The advantage gained is the removal of the lymph nodes lying on the first inch of the inferior mesenteric artery. Therefore although in theory the extensive operation is ideal, in practice the standard abdomino-perineal operation is carried out in many of these cases.

In spite of the fact that these principles of radical surgery have been recognised for several years, cases are still seen that have had only wedge excision which is often followed by early recurrence.

It is therefore a problem for consideration as to whether all cases of cancer of the colon should be treated by total colectomy.

Hepatic metastases. If the primary growth is removable it should always be extirpated, irrespective of the presence or absence of metastases in the liver. Although the case is obviously incurable every attempt should be made to remove the primary lesion for two reasons : (1) to stop the continual flow of cancer emboli to the liver ; (2) to prevent the gradual enlargement of the primary growth with involvement of other organs and attendant later obstructive symptoms, pain and sepsis. There is no doubt that provided the primary lesion has been removed a patient with hepatic metastases lives very much longer than a similar patient in whom only a short-circuit operation has been performed and the primary left *in situ*. It is unnecessary in patients with liver involvement to carry out the extensive resections as detailed for cases which appear curable. In the presence of one or a few hepatic metastases, partial hepatectomy is sometimes justifiable, either at the time of the original operation or later.

In cases which have appeared inoperable at the time of the first operation and have been treated either by entero-enterostomy or by a colostomy, it is often worthwhile performing a second laparotomy a few weeks after the first to see if by-passing of the faecal stream has resulted in such a diminution of sepsis and adherence that the lesion has become removable.

Surgical results (Table VIII). The first series of 892 cases was recorded by the British Empire Cancer Campaign and analysed by Colonel Harnett. The patients were treated in many different institutions by many different surgeons

The second series of 361 cases occurred in 190 general surgical hospital beds since World War II, and the patients were treated by a few surgeons who have striven to carry out a wider surgical excision than was the custom pre-war.

The main facts which emerge from these two series are that the resection rate has doubled, mortality rate has halved, and five-year survival rate has improved by fifty per cent.

As Dr. Arthur Allen of Boston has said, " curability bears a direct ratio to early diagnosis and treatment, and we must not dimish our efforts along educational lines."

TABLE VIII
CANCER OF THE COLON : RESULTS OF SURGERY

	Number of cases	Resections	Resection rate	Operation mortality	Five-year survival
			per cent.	per cent.	per cent.
Major London hospitals (Harnett B.E.C.C.) 1938-1939	892	311	34 8	18·3	31·8
Consecutive series in 190 surgical beds 1945-1956	361	232	65 7	8·6	48·0

Resection rate has doubled.
Mortality rate has halved.
Five year survival rate has improved 50 per cent.

There is therefore a great need for propaganda, both to the lay public and to the medical profession, of the urgency to suspect a colon disease, to recognize early symptoms and to ensure immediate reference for expert investigation and treatment.

I feel sure you would not wish me to close without bearing tribute to the many great names of those whose memory we revere in this field: Paul, Arbuthnot Lane, Moynihan, Grey Turner, to mention only a few.

I am indebted to Mr. Le Fanu, the librarian, and to the photographic department of the college, to Dr. Peter Hansell and the photographic department of Westminster Hospital, to Miss Hunt and Miss Akester of the photographic and medical artist department of the Royal Marsden Hospital, and to my colleagues and senior registrars, Mr. Gordon Ramsay, Mr. Allan Clain and Mr. Cyril Cooling for the unstinted help they have all given me.

I have endeavoured to call your attention to five points which I feel are of great importance in the study and treatment of the three main diseases of the large intestine.

1. More careful study during life, possibly by notification.

2. Education of the public and the profession in the symptoms of these diseases.

3. Increased clinical and radiological examinations with routine annual sigmoidoscopy after the age of 45.

4. I have stressed the importance of always endeavouring to restore intestinal continuity.

5. For all surgeons to practise more extensive radical operations for carcinoma of the colon.

I am reminded of a few lines from one of G. K. Chesterton's poems, in which I have only had to alter one word for my purpose:

" The wild thing went from left to right and knew not which was which
But the wild rose was above him when they found him in the ditch.

CARCINOMA OF THE CERVIX AT CHELSEA HOSPITAL FOR WOMEN 1933-56

FIVE-YEAR RESULTS OF TREATMENT 1933-51

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THE Chelsea Hospital for Women and the Royal Marsden Hospital * have (since 1944) conducted a joint clinic to which most of the patients suitable for radiotherapy are referred. This clinic has grown steadily and satisfactorily, and we believe that it provides a good example of the advantage to the cancer patient of cooperation between surgeon and radiotherapist.

A report covering the early years 1944–46 has already been published (Blaikley et al. 1952). We survey here the patients treated in 1933-56 and compare the results of treatment of carcinoma of the cervix in the period before the formation of the joint clinic (1933–43) with those obtained since (1944–51). Cases of cervical-stump carcinoma and carcinoma with associated pregnancy are reviewed separately. Carcinoma in situ, though mentioned, is not considered here in any detail.

Before 1944 surgery was the treatment of choice for carcinoma of the cervix, but patients unsuitable for operation were treated with radium at the Chelsea Hospital for Women by the surgeons without consultation with a radiotherapist; but, at the discretion of the surgeons, some patients were referred for X-ray treatment. Since the joint clinic was established in 1944, new patients are seen by both surgeon (J. B. B.) and radiotherapist (M. L.) together, and most of the new cases (83%) at the Chelsea Hospital for Women are referred to this clinic. Radiotherapy is given to these patients at the Royal Marsden Hospital, but if the number of cases is excessive, some of the radium insertions are undertaken at the Chelsea Hospital for Women. In most cases the patient is examined under anæsthesia, when the growth is staged clinically, a biopsy taken, and the final decision made as to the exact form of radiation treatment. Any decision necessitating a change in the plan of treatment is taken only after full consultation between surgeon and radiotherapist.

At the joint clinic, radiotherapy is regarded as the method of choice for treating nearly all cases of carcinoma of the cervix. The main indications accepted for surgery are:

(1) Recurrence after radiotherapy.

(2) Impracticability of adequate radiotherapy because of (a) anatomical abnormality preventing accurate insertion of radium, (b) pelvic sepsis, and (c) other tumours in the pelvis which require surgery.

* Until recently the Royal Cancer Hospital.

The mainstay of radiation treatment is radium, the X-ray therapy being used chiefly as a supplementary method.

Treatment

In principle, patients in stages I and II are treated with radium, a modified Stockholm technique being used, followed by supplementary X-ray therapy directed to the outer parts of the parametria and the side walls of the pelvis. Usually three radium treatments are given, the second a week after the first, and the third two weeks after the second. In stages III and IV cases X-ray therapy is applied before radium is used, and also when the carcinoma is bulky, fungating, or hæmorrhagic, the purpose being to shrink the growth and to control bleeding before embarking on the radium treatment.

Radium Technique and Dosage

In most cases 50–60 mg. of radium is inserted into the uterine cavity and 30–70 mg. into the vagina. In cases where the uterine source fills the length of the cavity and the vaginal sources completely cover all the area in the vault. At the same time as much radiation as possible is directed towards the side walls of the pelvis by using as large packets as possible with maximum distension laterally of the vaginal vault. Healthy tissues are protected by securely packing the radium in position.

At the Royal Marsden Hospital, the Manchester point A and B system is not used, because it cannot be applied to the highly individualised Stockholm technique which, with its variable radium sources and wide range of differing radium applicators, gives rise to a set of technical conditions which are the exact opposite to those

—	Patients seen	Treated	Not treated
New patients:			
1933–43	596	579	17
1944–51	437	421	16
1952–56	372	368	4
Total	**1405**	**1368**	**37**
Recurrent cases:			
1944–56	54	40	14
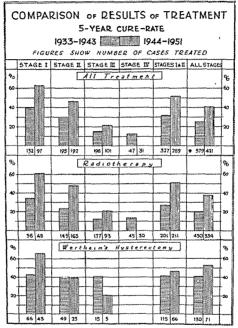
which the Manchester dosage system depends—i.e., the system of units of radium contained in special apparatus. It is, therefore, impracticable to prescribe dosage in terms of point A when using our Stockholm technique and equipment.

In these circumstances, information has to be obtained about the radium dosage delivered at each treatment to each patient. Fortunately it is possible, by using radiographic localisation techniques, to obtain the necessary information in the form of a single-plane radium dose distribution within the given patient's pelvis, whatever the combination of radium sources used (Lederman 1957). These individual distributions can be summated at the end of the series of radium treatments and a final distribution obtained showing the 6600r isodose surface within which all neoplastic tissue can be assumed to have been adequately irradiated. A distribution of this kind gives a reasonably accurate picture of the position and extent in a given pelvis of the zone embracing the cervix, vaginal tissues which require no further irradiation from any external sources that may be subsequently used.

To determine the radium dosage received by the peripheral pelvic tissues and the neighbouring normal viscera, direct measurement with a scintillation counting system can be undertaken. Since 1949 the dosage within the pelvis has been read directly with a probe

TABLE II—AGE-DISTRIBUTION OF 809 NEW CASES IN 1944-56

Age (yr.)	20-30	31-40	41-50	51-60	61-70	71-80	81-90	More than 90	Total
1944-1951 ..	4 (0·9%)	50 (11·4%)	91 (20·8%)	134 (30·7%)	115 (26·1%)	35 (8·0%)	8 (1·8%)	0	437
1952-1956 ..	13 (3·5%)	62 (16·7%)	86 (23·1%)	98 (26·3%)	80 (21·5%)	28 (7·5%)	3 (0·8%)	2 (0·5%)	372
Total ..	17 (2·1%)	112 (13·9%)	177 (21·9%)	232 (28·7%)	195 (24·1%)	63 (7·8%)	11 (1·4%)	2 (0·2%)	809

COMPARISON OF RESULTS OF TREATMENT
5-YEAR CURE-RATE
1933-1943 ▨ 1944-1951
FIGURES SHOW NUMBER OF CASES TREATED

* Includes 7 unstaged

type dose-meter (Lederman 1950) in all patients at each radium treatment. No attempt is made routinely to measure the dose received by the cervicovaginal neoplasm because the dose in this region is so high as to be of academic interest only. Attention is focused on the outlying pathways of spread of the disease and on the healthy tissues, which must not be damaged. The points selected for measurement are: the base of the bladder and both ureteric openings; the anterior wall of the rectum; and the inner and outer limits of both parametria.

Bladder and rectal complications have almost been eliminated since this instrument has been used. Experience shows that these do not develop if the bladder dose remains below 6000r and the rectal dose below 4000r.

X-ray Technique

Armed with the information derived from the radium distribution and the direct pelvic measurements, supple-mentary X-ray therapy can be given accurately to the areas ineffectively irradiated by radium. With X rays at 250-400 kV, tumour doses of 3000-4000r can be given in 4-6 weeks without severe systemic reactions or high rate of complications.

Patients in stages III and IV receive X-ray therapy first, applied to the whole pelvis. Subsequently radium is used in modified amount according to the response to treatment.

Material

Table I lists all cases seen in 1933-56, and in tables II and III all cases seen from 1944-56 are briefly reviewed in relation to age-incidence and tumour histology.

In 1944-48 there were no patients aged less than 30, in 1949-56 there were 17, of whom 5 were seen in 1954. The rising incidence of cervical carcinoma in young patients is quite striking and is shown in table II.

A biopsy specimen is taken from the cervix as a routine before treatment whenever possible. In an exsanguinated unfit patient X-ray treatment is given first and biopsy may be delayed until the radium insertion or the patient becomes fit for examination under anæsthesia. Serial biopsies during treatment have been undertaken during recent years, but it is difficult to know what significance to attribute to the information so derived.

In 27 cases treated in 1944–51 the biopsy was made-quate for diagnosis. 1 patient (stage II) has survived nine years; 3 patients with stage-III disease and 1 with stage-IV disease died of intercurrent disease within four years. The remainder all died of carcinoma within five years.

TABLE V—NEW PATIENTS NOT TREATED 1944-5

Stage	Number	Reason
I	0	Nil
II	3	All refused treatment
III	1	Refused treatment
IV	12	1 died before treatment was s; 1 unfit; 1 refused; in 9 the c was too advanced

TABLE III—HISTOLOGY 1944-56

Histology	New cases	Recurrent cases	Total
Squamous carcinoma	651	27	618 (78 6%)
Anaplastic carcinoma	68	6	74 (8 6%)
Adenocarcinoma	49	5	54 (6 3%)
Adeno- and squamous carcinoma	3	0	3 (0 3%)
Not confirmed	38	16	54 (6 3%)
Total	809	54	863

TABLE VI—RESULTS OF ALL TREATMENTS 1933-43
(596 patients seen, 579 treated)

Stage	No. seen	No. treated	Alive for 5 yr.	Lost in 5 yr.	5-yr survival-rate	Ab su r
I	132	132	51	22	38 6%	38
II	196	195	54	18	27-7 %	27
III	198	198	30	18	15-2%	15
IV	63	47	6	1	12 8 %	9
Unstaged	7	7	1	1		
I and II	328	327	105	40	32 1 %	32
All	596	579	142	60	24 5 %	23

Staging

The attempt was made to stage all the cases according to the international method adopted in 1950 (Heyman 1955). The staging in the three series shown in table IV is not strictly comparable in spite of reviewing the 1933–43

TABLE IV—STAGE-DISTRIBUTION OF TREATED PATIENTS

—	1933–43	1944–51	1952–56
Stage I	132 (23 0%)	97 (23 0%)	100 (27 2%)
Stage II	195 (34 0%)	192 (45 6%)	155 (42 1%)
Stage III	198 (34 0%)	101 (24 0%)	80 (21 7%)
Stage IV	47 (8 0%)	31 (7 4%)	33 (9 0%)
Unstaged	7 (1 0%)		
Total	579	421	368

cases; in particular, some cases in the 1944–56 group which formerly would probably have been placed in stage III are placed in stage II under the strict interpretation of the international staging

Untreated Cases

It is exceptional for a patient with advanced cancer of the cervix not to be offered some form of treatment even though purely palliative. Refusal to accept treatment or a moribund patient are the usual reasons for not giving treatment. In 1933–43, 16 patients were untreated because of advanced disease, and 1 patient with stage-II cancer refused, whilst as shown in table v, in 1944–51 16 patients were untreated, but only 9 of them because of the advanced stage of the cancer.

Results of Treatment

Tables VI–IX show the results of treatment in the years 1933–43, and tables X–XIII the results obtained after setting up the Joint Consultation Clinic (1944–51). The results for both periods are compared in the accompanying figure.

Tables IX and XIII show the results of treatment other than Wertheim's hysterectomy or radiotherapy. These include cases operated on for conditions other than carcinoma when the diagnosis of malignancy was not made until histological examination. In 1944–51 there were also 3 anterior exenterations and 1 total exenteration following radiotherapy; these are included in table XIII. The other treatments are too numerous to list individually but include 5 cases of hysterectomy and bilateral salpingo-oöphorectomy followed by radiotherapy, and 3 cases of radiotherapy and bilateral iliac

lymphadenectomy. Pelvic exenterations are don Chelsea Hospital for Women mainly for recurrent c noma, and often where the primary treatment has given elsewhere. In 1944–51 16 exenterations were d 9 total and 7 anterior. Only 3 of these patients surv 5 years.

Radiotherapy

The results of radiotherapy in 1933–43 were bad are a direct reflection on the poor technical stand of radium therapy then existing and the complete of correlation between radium and X-ray therapy, results of surgery were considerably better than th obtained by radiotherapy, 40·9% against 26·4% stages I and II. The results of Wertheim's hysterect were reasonable for that time.

The period 1944–51 was characterised by a very g improvement in the radiotherapy results, the surv rates being doubled from 19 5% to 38%. In the s: period the surgical results also improved (from 40 to 53 5%) but to a much less striking extent—e.g., stage I and stage II radiotherapy results increased fr 26 4% to 51·2%, whereas in the same period the rea of Wertheim's hysterectomy for the same stages impro from 40 9% to 56·1%.

One feature of the radiotherapy results is, on t surface, disturbing and merits special comment

TABLE VII—RESULTS OF RADIOTHERAPY 1933-43
(430 cases)

Stage	No. treated	Alive for 5 yr.	Lost in 5 yr.	5-yr. su viral-ra:
I	56	19	9	33 9 %
II	145	34	12	23 5 %
III	177	21	17	12 4 %
IV	45	6	1	22 %
Unstaged	7	1	1	
I and II	201	53	21	26 4 %
All	430	81	41	18 7 %

TABLE VIII—RESULTS OF WERTHEIM'S HYSTERECTOMY 1933-43
(137 cases)

Stage	No. treated	Alive for 5 yr.	Lost in 5 yr.	5-yr. su viral-ra:
I	54			
II	68	23		
III	13			
I and II	122			
All	137			

namely, the relatively low survival-rate in stage-I cases (1944–51). The 60 4% survival-rate obtained in *stage-I cases is considerably below the 70% (or better)* rate reported by other centres. A detailed analysis of our stage-I cases gives the following information:

(1) There is a definite selection of stage-I cases for radiotherapy Thus, of 97 cases seen in 1944–51 only 48 were treated primarily by radiotherapy. Many of these patients were referred only because they were unsuitable for radical surgery because of age, general condition, or the anaplastic nature of the tumour.

(2) Of the 48 cases treated by radiotherapy 13 (27%) were not referred to the Joint Clinic until after some part of their radiation treatment had already been given In this group there were only 5 survivors after five years—i.e., 38·4% (cf. 38 6% for 1933–43). Of the remaining 35 patients who were treated from the outset by the Joint Clinic, 24 .... with

disease and 7 of cancer. In all these 7 cases the patients had either anaplastic or large bulky tumours

Wertheim's Hysterectomy

In assessing the results of Wertheim's hysterectomy the effect of radiotherapy given before or after operation must be considered In 1933–43, 64 of 170 patients received radiotherapy, and in 1944–57, 56 of 71 patients also received radiotherapy.

Table XIV gives the results of Wertheim's hysterectomy in 1933–51 in detail according to the type of radiotherapy

TABLE IX—RESULTS OF TREATMENT OTHER THAN A RADIO-THERAPY OR WERTHEIM'S HYSTERECTOMY IN 1933–43 (19 cases)

Stage	No treated	Alive for 5 yr.	Lost in 5 yr.	5-yr survival-rate
I	10	4	2	
II	1	1	0	
III	6	0	0	
IV	2	0	0	
All	19	5	2	26 3%

TABLE X—RESULTS OF ALL TREATMENTS IN 1944–51 (437 patients seen, 421 treated)

Stage	Total seen	No. treated	5-yr survival	Lost	Inter-current disease	5 yr survival-rate	Absolute survival rate
I	97	97	61	1	6	62 9%	62 9%
II	195	192	89	1	6	46 1%	45 6%
III	102	101	21	0	6	20 8%	20 5%
IV	43	31					
I & II	292	289	150	2	12	51 9%	
All	437	421	171	2	18	40·6%	39 2%

given. The number of cases was too small to compare the results before and after 1944. There was a great radium was stage I and y alone was 34%, but if radium were used preoperatively, the rate rose to 56%

stage-I patient and 1 stage-II patient died of cancer within that time The lymph-nodes were free from growth in the first 2 cases, but were involved in the last 2

Carcinoma of Cervical Stump

In 1933–56 38 previously untreated cases of stump carcinoma were seen. The total number of new cases

of carcinoma of the cervix during this period was 1405, giving an incidence of 2 7%, the incidence having risen from 1·9% before 1944 to 3 3% since that time, as shown in table xv.

Age (yr.)	..	30–39	40–49	50–59	60–69	70 or more
No. of patients	..	2	6	16	11	3

TABLE XI—RESULTS OF RADIOTHERAPY 1944–51 (334 cases)

Stage	No. treated	5-yr. survival	Lost	Inter-current disease	5 yr. survival-rate
I	48	29	1	5	60 4%
II	163	79	—	5	48 5%
III	93	19	—	6	20 4%
IV.	30	—	—	—	
I and II	211	108	1	10	51·2%
All	334	127	1	16	38 0%

TABLE XII—RESULTS OF WERTHEIM'S HYSTERECTOMY 1944–51 (71 cases)

Stage	No treated	5-yr. survival	Lost	Inter-current disease	5-yr. survival-rate
I	43	28	—	1	65 1%
II	27	9	—	—	39 1%
III	5	1	—	—	
I and II	66	37	—	1	56 1%
All	71	38	—	1	53 5%

Parity.—Of the 38, 7 (18%) were nulliparous; the distribution of pregnancies was as follows:

No of pregnancies	Unknown	Nil	1	2	3	4	5	6
No. of patients	2	7	9	7	6	2	2	3

follows:

No of cases	Pathological Findings
15	Fibroids
1	
1	
1	
1	
1	Fibroma
1	Tuberculous pyosalpinx
12	Not recorded

4 of the 12 patients whose pathology was not recorded stated that hysterectomy for fibroids had been performed.

Time Intervals.—The time-intervals between the hysterectomy and the diagnosis of carcinoma were as follows:

Time (yr)	No of Cases	Time (yr)	No of Cases
Less than 1 year	3	21–25	9
2–5	3	26–30	2
6–10	7	31–35	2
11–15	4		1
16–20	6		2

Results of Treatment.—In 1933–43 11 patients were treated 2 in stage I by Wertheim's hysterectomy,

TABLE XIII—RESULTS OF TREATMENT OTHER THAN WERTHEIM'S HYSTERECTOMY OR RADIOTHERAPY 1944–51 (16 cases)

Stage	No. treated	5-yr survival	Lost	Inter-current disease	5 yr survival-rate
I	6	4	—	1	
II	9	1	1	—	
III	0	—	—	—	
IV.	1	—	—	—	
All	16	6	1	1	37 5%

TABLE XIV—RESULTS OF WERTHEIM'S HYSTERECTOMY 1933–51

| Method | No. of cases | | | | 5-yr cure | | | |
| | Stage | | | Total | Stage | | | Total |
	I	II	III		I	II	III	
Wertheim alone	46	30	5	81	16	10	0	26
Radium followed by Wertheim's hysterectomy	48	29	11	88	30	13	6	49
High-voltage therapy after operation	6	5	3	14	5	2	1	8
Preliminary radium and high-voltage therapy after operation	9	8	1	18	5	3	0	8

9 by radiotherapy 2 patients treated by radiotherapy survived five years and were still alive after ten years In 1944–51 18 patients were treated : 1, a stage-I case, treated by Wertheim's hysterectomy, has survived ten years ; the remaining 17 were treated by radiotherapy, and 5 of these survived five years The results are shown in table XVI.

Carcinoma of Cervix and Pregnancy

In 1933–56 6 cases of carcinoma of the cervix in association with pregnancy were recorded, giving an incidence of 0 43%. Table XVII shows details of these cases

Carcinoma-in-situ of Cervix

The diagnosis of carcinoma-in-situ was not in use before 1950 ; since then 17 cases have been recorded, more than half of them being diagnosed on pathological examination following either hysterectomy or amputation of the cervix None of these patients has developed invasive carcinoma to date

Conclusions

Since the inception of the Joint Clinic in 1944 the results of treatment of carcinoma of the cervix have greatly improved. The five-year survival-rate for patients treated by Wertheim's hysterectomy has risen from 40·8% for the years 1933–43 to 53 5% for 1944–51, and

TABLE XV—INCIDENCE OF CARCINOMA OF CERVICAL STUMP

Period	Total patients seen	Stump carcinoma	Incidence
1933–43	596	11	1 9%
1944–56	809	27	3 3%
Total	1405	38	2 7%

during the same periods the survival-rate for stage-I and stage-II cases treated by radiotherapy have risen from 26 4% to 51·2%. The two groups cannot be directly compared, because the hysterectomy group is selected as regards stage-II cases , in spite of this the results of radiotherapy are similar

TABLE XVI—RESULTS OF TREATMENT OF STUMP CARCINOMA

| Stage | 1933–47 | | 1944–53 | |
	No. treated	Alive 5 yr	No. treated	Alive 5 yr.
I	5	0	3	3
II	4	2	8	2
III	1	0	5	1
IV	1	0	2	0
All	11	2	18	6

The results of radiotherapy in stage-I cases a interest because the overall five-year survival-rat 60 4% is disappointingly low and show s less improver than in the other stages : it has to be compared with figure of 65·1% obtained by Wertheim's hysterect However, for the radiotherapy group where the t ment was exclusively planned and given at the Clinic, the five-year survival-rate is 69% If all where death took place from intercurrent disease excluded, the rate is 77% compared with 67% Wertheim's hysterectomy. It therefore seems radiotherapy is not only undoubtedly the correct t ment in inoperable cases but also usually the prefe treatment for operable cases.

From the small number of cases reviewed it also s that the results of Wertheim's hysterectomy are gre improved by preoperative treatment with radium.

The difference in results obtained according a whether or not there has been full cooperation bet surgeon and radiotherapist is most striking. T

TABLE XVII—CARCINOMA OF CERVIX ASSOCIATED PREGNANCY 1933–56

Case no.	Year	Age (yr.)	Stage	Duration of pregnancy (weeks)	Pathology	Treatment	Result prese
1	1935	36	I	10	Squamous	Wertheim	Alive &
2	1948	33	I	18	,,		Died in
3	1948	36	I	16	,,		Alive &
4	1953	40	II	16	,,	Radiotherapy	Died in
5	1954	41	II	12	,,	Radiotherapy	Alive &
6	1956	31	I	14	Anaplastic	Radiotherapy	Alive &

seems to be little doubt that, if the best results are t obtained, this type of cooperation must be forthcom for every case of carcinoma of the cervix

Finally, we believe that the results obtained w radiotherapy by the joint clinic are probably as good can be achieved by our present methods, but furt improvement should be possible with still greater ind dualisation of technique and the increased availabilit apparatus in the supervoltage or megavoltage range

Summary

The cases of carcinoma of the cervix at the Chel Hospital for Women in 1933–56 are reported

A joint consultation and treatment clinic , under t care of a surgeon of the Chelsea Hospital for Women a a radiotherapist of the Royal Marsden Hospital, w established in 1944. Since that time patients treated radiotherapy have been transferred to the Roy Marsden Hospital. The five-year results for 1933– are compared with those for 1944–51 and discussed.

The cases of stump carcinoma and of carcinoma ass ciated with pregnancy are also reviewed.

We wish to thank all those surgeons of Chelsea Hospital f Women, Lewisham, The Mayday, and other hospitals f their cooperation and encouragement in referring patien to the Joint Clinic.

Our thanks are also due to Mr. A. J. Bailey for t illustration

REFERENCES

Blaikley, J B , Lederman, M , Leis, Y L T (1952) *Lancet*, ii 950.
Lederman, M (1950) *Brit J Radiol* 23, 430
— (1954) *Acta Un int Cancr* 10, 385.
Heyman, J (1955) Annual Report of Treatment of Carcinoma of th Uterus, vol 10, p 33 Stockholm

The Use of Myleran and Similar Agents in Chronic Leukemias

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I. INTRODUCTION

The treatment of leukemia is still dominated by two facts: (1) palliation alone is possible, and (2) resistance to all known methods of treatment eventually develops. Thus leukemia remains a fatal disease. Lissauer (1865) was the first to record successful palliation in chronic leukemia by means of arsenic, which is still used. Radiotherapy was first used by Senn (1903), and remains the basis of present-day treatment: the use of benzene was introduced by Koranyi (1912) and extended by Kalapos (1935). All of these drugs were principally of value in chronic myeloid leukemia. The more recent drugs include nitrogen mustard and its allies (Goodman *et al*, 1946), urethane (Paterson *et al.*, 1946, 1947), triethylenemelamine (Karnofsky *et al.*, 1951), Myleran (Haddow and Timmis, 1953; Galton, 1953a), 6-mercaptopurine (Burchenal *et al.*, 1953), 1,3-bis(ethyleneimino-

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sulfonyl)propane (Paterson and Kunkler, 1954), and deacetylmethylcol-chicine (Moeschlin *et al.*, 1954). Of these drugs only the nitrogen mustards and triethylenemelamine have been widely advocated in the treatment of chronic lymphatic leukemia, though urethane has been sometimes successful (de Vries, 1953). Meanwhile radiotherapy techniques have been refined and extended. Lawrence (1954) introduced radioactive phosphorus which is a most effective form of radiotherapy in both types of chronic leukemia (Lawrence *et al.*, 1949; Lawrence *et al.*, 1948), and whole-body external radiotherapy was equally useful (Osgood, 1951; Osgood and Seaman, 1952). Radioactive colloidal gold (Fellinger *et al.*, 1956) and radio-active colloidal phosphorus (Hill *et al.*, 1954) have also been tried. Tri-ethylene-thiophosphoramide (Thio-tepa) is a modification of triethylene-melamine that has been used successfully in the treatment of chronic leukemia (Zarafonetis *et al.*, 1955).

The relationship between the acute and chronic leukemias is not clear and it is therefore of interest that the methods of treatment just listed were mostly found to be valueless in the acute leukemias. 6-Mer-captopurine is a notable exception. Effective palliative therapy in acute leukemia dates from the work of Farber *et al.* (1948) with folic acid antagonists. Subsequently, remissions were obtained with corticotropin, cortisone, and hydrocortisone (Pearson *et al.*, 1949). 6-Mercaptopurine remains the most useful of a number of purine antagonists tested (Silva *et al.*, 1954). The folic acid antagonists are not useful in the chronic leukemias. It is usual to distinguish the group of general and antimitotic cell poisons effective in chronic leukemia from the nutritional antagonists that are useful in acute leukemia. However, the distinction is not abso-lute, since 6-mercaptopurine is active in both the acute and chronic forms, and urethane, which perhaps acts as an antimetabolite by in-hibiting thymine synthesis (Boyland and Koller, 1954), is inactive in the acute disease. Furthermore, it is not known whether purine or folic acid antagonism are the properties responsible for the antileukemic activity of purine and folic acid antagonists. But simple destruction or suppression of leukemic cells does not account for the remissions of acute leukemia, for bone marrow aplasia brought about by the antimitotic poisons listed above does not lead to remission; and hormone-induced remissions are not always preceded by an aplastic phase. In acute leukemia remission seems to depend on some event other than simple growth suppression. But in the chronic leukemias, the antimitotic poisons and ionizing radia-tions that are the most effective therapeutic agents are all powerful bone marrow depressants. Yet the folic acid antagonists also damage bone marrow but are of little value in the chronic leukemias (Silva *et al.*, 1954). X-irradiation of the spleen in chronic myeloid leukemia is followed by

changes in the bone marrow that cannot be a direct result of irradiation damage (Gunz, 1953). Thus the mechanism of action of therapeutic agents in both acute and chronic leukemias remains obscure.

We learn from medical history that for any disease the number of remedies available is inversely proportional to their efficacy. The present-day version of polypharmacy in the treatment of leukemia reminds us that we are far away from an understanding of the fundamental disturbance underlying this disease. In the following account of the clinical use of Myleran it is to be understood that we are dealing with no new principle in the treatment of chronic leukemia, but only with an addition to the growing list of palliatives. Myleran merits special description because it possesses certain advantages in the handling of chronic myeloid leukemia which should secure for it at least a temporary place in management.

II. PROBLEMS OF CLINICAL ASSESSMENT

1. The General Problem

When the clinician carries out the first trials with a new remedy, the problems he faces are very different from those of the experimental biologist. The latter can restrict his inquiry to the simplest issues, though almost always these prove to be infinitely more complex than he might at first have thought. He can isolate a single factor and by correct use of controls, can study its behavior under a variety of conditions. By using pure genetic strains of organisms, he can achieve a high degree of uniformity in the behavior of his material, and can reach greater precision by using more animals. When small animals are used, their sex, weight, and age can be selected as desired and their diet can be varied at will. The clinician on the other hand has to take his patients as they come, members of a mongrel species, with varying social backgrounds, occupational, and dietary habits and different histories of previous illness. Therapeutic aims vary. One may, for example, study the action of a new drug on a particular symptom, as did Withering in treating dropsical patients with digitalis, or Sir James Mackenzie who used the same drug in auricular fibrillation. In such studies the patient serves as his own control and the method may be compared with that of the experimental pharmacologist. The patient himself is also the control in the assay of specific nutritional factors whose effects on the clinical and laboratory manifestations of relapse are observed. Lind's studies of scurvy and Minot and Murphy's work on pernicious anemia are classic examples. The assessment of remedies in acute infectious diseases with a variable mortality requires another approach, that of the controlled clinical trial in which patients who are to receive the treatment to be tested are

selected at random from a group; the remainder serve as controls and the results of treatment are analyzed with respect to specific features such as influence on mortality, on incidence of complications, or on speed of recovery. This method was used in the early trials of sulfonamides in puerperal sepsis and of all the antibiotics. It is the basis of the more complex trials of antibiotics and chemicals in chronic infections such as leprosy and tuberculosis, or of immunizing agents in diphtheria, pertussis, influenza, or poliomyelitis.

Remedies for incurable diseases may be assessed in various ways. In very chronic maladies, different forms of symptomatic treatment may be tried successively and their comparative efficacy may be worked out. Thus the effects of aspirin and cortisone in relieving the pain of rheumatoid arthritis have been recently studied (Medical Research Council, 1954). The ability to relieve rigidity and tremor in paralysis agitans by drugs like diparcol and artane was tested in the same way. These remedies are strictly palliative. The survival of diabetic patients has been prolonged by the discovery of insulin which controls faulty carbohydrate metabolism. Insulin, like vitamin B_{12} in pernicious anemia, is an effective *substitute for an essential substance that the diabetic cannot make. In both diseases the cause remains unknown.* Subacute bacterial endocarditis is a fatal disease of known cause. The value of penicillin was demonstrated by extended trials in which controls were not used because of the inevitably fatal outcome in the untreated case. The justification for trying penicillin was that the responsible organisms were known to be sensitive *in vitro*. The trials of streptomycin in tuberculous meningitis were analogous.

In cancer, the justification for surgical extirpation as early as possible is the belief that the growth begins locally. In the case of cancer arising in the more accessible sites the survival rates support this belief but tell us little of the conditions under which the surviving patients live. Many of them linger on for several years more or less incapacitated by recurrence. A more realistic measure of therapeutic success is the recurrence-free rate. Once the disease has become generalized, treatment may not prolong life but in some instances may render it more tolerable. Such treatments are castration, hormone therapy, adrenalectomy, or hypophysectomy for mammary and prostatic cancer. From the standpoint of therapeutic assessment, leukemia may be regarded as a malignant process that has become generalized, and will now be discussed.

2. Special Problems of Clinical Assessment in Chronic Leukemias

In the acute leukemias, survival has been significantly prolonged by modern methods of treatment; yet the disease remains fatal. In the

chronic leukemias, either no lengthening of life has resulted from treatment (Minot et al., 1924; Minot and Isaacs, 1924; Hoffman and Craver, 1931; Shimkin et al., 1951, 1953), or a meager prolongation of survival has required the most refined statistical analysis to reveal it and cannot be attributed with certainty to any particular treatment or method of using it. "Recent advances in internal medicine, new anti-biotics, adequate whole blood etc., are all uncontrolled factors which should help improve the patient's survival. The success indicated (referring to patients treated by 'titrated, regularly spaced total body-radio-active-phosphorus, or roentgen irradiation,' Osgood and Seaman, 1952) must be ascribed to the total treatment of the disease and its complications (without divided responsibility) using all the agents available and not to any single agent or part of the treatment" (Tivey, 1954). Tivey has demonstrated a skewed distribution of survival in all series of cases of chronic leukemia analyzed. The mean survival is a misleading parameter because small numbers of patients survive far longer than the majority. For both forms of chronic leukemia the commonest survival from onset is 2.6 years; but some patients may survive for many years (McGavran, 1938; Moffit and Lawrence, 1949). The shape of the survival curve of chronic leukemia is similar to that of malignant disease. When very small numbers of patients are treated, short or long survival of most of them may reflect the unrepresentative composition of the series rather than the effect of treatment employed. Tivey has described a method whereby the survival curve of a group of patients can be predicted with some accuracy while some of the patients are still living. If a new remedy is thought capable of prolonging life, this method might be useful if the treatment is being carried out properly. This is most unlikely during the first years of trial. As long as new methods of treating chronic leukemia represent only minor improvements of existing ones, statistical methods will be necessary to reveal increased survival, but the effect of a radical therapy would quickly become evident to the clinician. The use of available remedies is better justified by their capacity to relieve symptoms and so to increase the tolerability and efficiency of life.

The evaluation of symptomatic benefit largely concerns the patient as an individual, though in certain instances controlled studies on groups are necessary, for example in the assay of remedies against sea sickness, when one group is treated with the remedy under trial, another with an existing remedy with which it is to be compared, while a third group receives a "dummy" preparation. In leukemia these elaborations have no place. The symptoms are successfully relieved or they are not. The principle is simple. The practice is complicated by factors peculiar to leukemia that will be considered shortly. If the capacity to relieve symp-

toms is the sole object of study, then there would be no justification for
applying the new remedy in symptomless cases, though the investigation
of capacity to prevent relapse by suitable maintenance therapy in a
patient already treated would be legitimate. If on the other hand a new
remedy is to be tested for its ability to prolong life, then the presence or
absence of symptoms will be irrelevant and the assessment of results will
depend more on a survey of the group as a whole than on the responses
of the individual patients.

Now in the acute leukemias, experience has shown that the signs of
relapse following a spontaneous or drug-induced remission are inde-
pendent of the return of symptoms. Abnormalities are found in the blood
and bone marrow weeks before the return of symptoms. These abnormali-
ties can be corrected by treatment. A child with acute leukemia may
remain symptom-free for over a year and may have relapsed two or three
times, each relapse responding to appropriate treatment. Such treatment
is justified by the increased survival it has brought about. In the chronic
leukemias, abnormalities also appear in the blood and bone marrow long
before the onset of symptoms and the effect of treatment designed to
correct these abnormalities regardless of the presence of symptoms has
been described by Osgood and Seaman (1952). The difficulties of inter-
preting the results in terms of the treatment given have been noted above
(Tivey, 1954). The differences from the results of conventional therapy
in which treatment is designed only to relieve symptoms do not yet
appear sufficiently important to justify the routine treatment of patients
without symptoms whose disease has been accidentally discovered.

3 The Three Stages of Chronic Leukemia

The response to all forms of therapy depends as much on the stage of
the disease as on the particular treatment employed (Silva et al., 1954).
When a new form of therapy is first described it is therefore very neces-
sary to define the clinical stage of each case in which it is used

Although chronic leukemia may develop within a few months of the
finding of a normal clinical and hematological picture (case "J. A ,"
Meyer et al., 1952) it is probably more usual for the symptomless induc-
tion phase to last several years. This is known from cases in which chronic
leukemia is discovered accidentally during routine examination for some
unrelated condition; and the development of leukemia has been followed
in persons exposed to leukemogenic agents. Thus, Maingot et al. (1938)
observed the onset of chronic myeloid leukemia over a period of 13 years
in a radiologist's assistant, while Moloney and Lange (1954) and Lange
et al. (1954) described the increased incidence of leukemia in the Japanese
populations exposed to ionizing radiations emitted from atomic bombs.

In this case the incidence of frank leukemia increased for 6 years after the bombs were dropped, and subsequently declined. In the statistical studies cited above, the survivals were calculated from "time of onset." This includes symptomless cases discovered by routine examination as well as those in which onset was dated from time of first appearance of symptoms, but must have preceded it by an unknown period, possibly several years. It would be instructive to analyze the two groups separately. A higher proportion of the longer-surviving cases might be found in the symptomless group An unselected series might thus be biased in favor of long survival if it contained many symptomless cases, as could happen in any community where routine blood counts were performed frequently. It is certain, for example, that routine blood examinations are more frequently carried out in United States than in British clinics.

Sometimes the first symptoms appear suddenly in apparently healthy persons. Splenic infarction or priapism may be the first indications of chronic myeloid leukemia, while the discovery of an enlarged lymph node is a common way for chronic lymphatic leukemia to present itself. These events may be regarded as clinical accidents leading to early recognition of leukemia that might otherwise have remained symptomless for months or even years. As far as response to treatment is concerned these cases are closer to the preceding group than those now to be discussed.

Insidious onset of ill health is common in chronic myeloid leukemia, and may be tolerated for long periods before some added symptom such as abdominal pain or the onset of sweats leads the patient to seek medical advice. These symptoms are usually relieved completely by treatment and when they recur yield to further treatment. Whatever method is employed, a stage is reached when symptoms are only partially relieved, when little or no regression of enlarged liver, spleen or lymph nodes takes place, and hematological changes, especially increase in hemoglobin, are less marked or are of only short duration. If the treatment is changed, better response may be obtained, and the patient may remain well, sometimes for several years But control is precarious, the blood picture becomes gradually more "blastic" or acute myeloblastosis suddenly appears. Until recently myeloblastosis was regarded as a rapidly fatal event but temporary response has been obtained with 6-mercaptopurine and deacetylmethylcolchicine (see below).

In chronic lymphatic leukemia the course of the disease differs from that in the myeloid form The symptomless phase may last several years. A patient without complaints may have generalized lymph node enlargement, a high lymphocyte count, and a bone marrow heavily infiltrated with lymphocytes. Apart from symptoms resulting from local pressure of enlarged nodes, such patients commonly remain fit until they succumb

to infection, which is frequent in lymphatic leukemia, or to anemia (Wintrobe and Hasenbush, 1939). The infections usually respond to antibiotics, but the anemia, which may arise in different ways (Berlin *et al.*, 1954) does not respond to radiotherapy or chemotherapy with the regularity of the anemia of chronic myeloid leukemia. When it is frankly hemolytic it may respond better to such nonspecific remedies as splenectomy (Berlin, 1951; Wintrobe *et al.*, 1954) or to cortisone. One patient has been well controlled for over three years on cortisone alone; attempts to give triethylenemelamine twice resulted in hemolytic crises (Nabarro, 1955). Thrombocytopenia, associated with depletion of the bone marrow of megakaryocytes is a frequent hazard in this disease and hemorrhage resulting from it is a common cause of death Very occasionally the platelet count improves after treatment with radiotherapy or chemotherapy but it is more usual for treatment to depress the platelet count still further. Some authorities therefore regard thrombocytopenia as a contra-indication to treatment with bone marrow depressants (Silverberg and Dameshek, 1952).

In both forms of chronic leukemia it is thus extremely difficult to decide at what stage radiotherapy or chemotherapy should be given or to establish criteria for judging their value. If the patient has no symptoms what exactly is the treatment doing? In lymphatic leukemia both radiotherapy and chemotherapy will reduce the lymphocyte count in the blood and bone marrow and the size of lymph nodes and spleen, but if there is no anemia, no pressure effects from lymph nodes or spleen, and the patient is well, what is the significance of such treatment? Since the untreated patient commonly continues in good health for a number of years, what is the evidence that treatment defers the onset of symptoms, prevents anemia or thrombocytopenia? When these catastrophes supervene, the specific remedies are admitted by most to be relatively ineffective (Silva *et al.*, 1954; Wintrobe *et al.*, 1954). Two opposing views are held. Osgood favors treatment as soon as the disease is diagnosed and feels that subsequent treatment should be designed to maintain the leucocyte count below an arbitrary level without regard to the patients' condition. In this way he believes that patients are kept in better condition and they live longer than when conventional methods are used (Osgood and Seaman, 1952) Nevertheless, of 102 of his patients with lymphatic leukemia, 56 had survived 3 years or more before they had begun to receive the method of treatment he advocates (Fig. 3, Osgood and Seaman, 1952), and we have already seen from Tivey's work that the median survival from onset in unselected cases was 2.6 years. The opposite view is the strictly conventional one according to which patients are not treated until they complain of feeling unwell. This means that

treatment is begun at varying stages. Some patients are more sensitive to minor symptoms than others; in a disease-conscious community, a patient who finds a single enlarged node quickly discovers other things wrong with himself, real or imagined, whereas a less observant patient may not notice that he has many enlarged lymph nodes; a patient in a sedentary occupation may feel well with a hemoglobin level of 9 g. per 100 ml., whereas a manual laborer will feel unwell with 12 g. per 100 ml. Much depends on the attitude of the patient and of the physician. Referring to chronic lymphatic leukemia, Wintrobe *et al.* (1954) state "some would question the need or even the desirability of therapy as long as no anaemia is present and tumour masses are not so large as to be troublesome. Our own attitude is to treat but to err on the side of too little rather than too much. A number of patients managed in this way, felt better and gained weight."

It is thus clear that the indications for treatment and the criteria of success vary widely. This accounts for much of the difficulty in comparing different accounts of clinical trials of new remedies. When the author speaks of "remissions" lasting so many months or years, it is necessary to discover what "remission" means to him. Some writers distinguish between "clinical" and "hematological" remissions, the former presumably being more pleasing to the patient, the latter to the doctor. In the acute leukemias, this distinction is of value but in the chronic forms varying degrees of hematological abnormality are quite compatible with excellent health. Kurrle (1955) therefore speaks of "period of control" rather than of remission. In the practice of the writer and his colleagues, symptomless patients with either form of chronic leukemia are not treated. But they are examined at two or three-monthly intervals and their blood counts are charted from their first visit. Particular attention is paid to the hemoglobin level and to the platelet count. If the hemoglobin level begins to fall, and causes for this other than leukemia are excluded, the patient is asked to attend more frequently, and if the fall continues treatment is begun even in the absence of symptoms. In chronic lymphatic leukemia a falling platelet count is also regarded as an indication for treatment because whatever form of therapy is used, further depression is common in the first three months although subsequent increase in numbers of platelets above the pretreatment level often occurs. In chronic myeloid leukemia there is a risk of priapism when the platelet count exceeds 1,000,000 per cu. mm. (Galton and Till, 1955). Continued increase in platelet count is therefore regarded as an indication for treatment in symptomless patients. In symptomless patients with both forms of leukemia, neither the leucocyte count nor the bone marrow picture are considered to give much help in deciding the need for treat-

ment, though the leucocyte count is very important in the maintenance therapy of patients who have already received treatment. In this respect our practice is similar to that of Osgood, but we set no arbitrary limit for controlling the leucocyte count.

4. Relevance of Clinical Results to Fundamental Research

The ability to relieve symptoms or to prolong life may not be closely related to the fundamental disease process of leukemia. Diphtheria antitoxin relieves symptoms and prevents the lethal effects of toxin but does nothing to eradicate the infection. In the chronic leukemias especially, most of the symptoms are secondary to excessive production of leucocytes and are relieved, by agents which interfere with this. The cause of the abnormal production may itself remain unaffected. It is thus difficult to know how far the results of clinical trials should influence the research worker in his efforts to discover more effective antileukemic agents. Improvements in the chemical structure of effective compounds that might be suggested by the results of clinical trial could lead to improvements in therapy analogous to those associated with refinements in the preparation of antitoxins, without coming nearer to the basic abnormality. All the most useful agents developed in recent years have arisen directly from basic studies in growth inhibition and growth metabolism but the disordered growth of hemopoietic tissue which is the most obvious characteristic of leukemia may prove to be secondary to a disturbance unconnected with growth.

III. The Discovery of Myleran

Timmis (1948) and Haddow and Timmis (1951) showed that the property of biological alkylation could be conferred on the ethyl groups in diethylarylamines by the introduction of aromatic sulfonic acid ester groups. This property had been previously associated only with the di-2-chloroethyl group characteristic of the nitrogen mustards. One of the first compounds active in transplanted tumors was (I)

(I)

The dimesyloxyalkanes, of which Myleran is a member, represented the final step in the simplification of the bifunctional molecule with retention of the sulfonic acid ester groups.

Myleran (II) was soon found to be a powerful inhibitor of the transplanted Walker carcinoma in the rat (Haddow and Timmis, 1953).

(II) $CH_3SO_2O(CH_2)_4OSO_2CH_3$

Its effect on the growth rate and on the blood cells of normal rats was found by Elson to differ markedly from that induced by numerous nitrogen mustards (Elson, 1955). The lymphocytes were irregularly affected but the numbers of neutrophils fell progressively for 12 days after a single dose. A progressive anemia developed lasting for 3 weeks. These changes result from a prolonged action on the bone marrow affecting all elements, entirely different from that caused by the nitrogen mustards, and of a type not previously described (Elson et al., 1955). Myleran damages the gonads of the rat (Bollag, 1953a) and if given late in pregnancy renders the offspring sterile (Bollag, 1954a). Earlier in pregnancy quite large doses do not interfere with its course and the F_1 and F_2 generations are apparently healthy (Galton and Till, 1953).

Early clinical trials of Myleran and one of its analogues GT 78 (III)

(III) $CH_3SO_2O(CH_2)_6OSO_2CH_3$

in cases of advanced mammary and bronchial carcinoma, malignant melanoma, neuroblastoma, seminoma, angiosarcoma, fibrosarcoma, and Hodgkin's disease gave negative results. But two features were noted: first, no patient complained of side effects following oral administration; second, in every case profound neutropenia ensued (e.g. Figs. 2 and 3, Galton, 1953b), but the lymphocytes were scarcely affected.
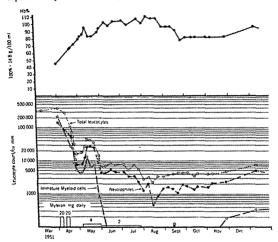
These facts suggested the use of Myleran in myeloid leukemia, and the first patient, who was no longer responding well to x-rays was treated in September, 1950 (case 1, Galton, 1953a). Other reports soon appeared (Bollag, 1953b; Turesson, 1953; Hansen, 1954; Wilkinson, 1953; Klima et al., 1954; Petrakis et al., 1954; Levin et al., 1954; Louis et al., 1954; Moloney and Takashi, 1954; Wintrobe et al., 1954; Galton and Till, 1955; Kurrle, 1955; Videbaek, 1955; Bernard et al., 1955).

IV. ACTION OF MYLERAN IN CHRONIC MYELOID LEUKEMIA

1. Administration and Dosage, Toxicity and Side Effects

Although Myleran can be administered intravenously (Petrakis et al., 1954), for general use the oral route is better. In nonleukemic subjects single oral doses of 2-3 mg. per kilogram of body weight have been given. Such doses always caused profound depression of the blood monocyte and neutrophil counts, moderate reduction in numbers of platelets, and inconstant changes in the numbers of lymphocytes. The first changes in the blood count were evident after 1 week and continued for 3 weeks

after which brisk recovery followed, accompanied by the appearance of immature myeloid cells in the blood. These changes indicate direct toxic action on the bone marrow. An analogue of Myleran, 1,4-dimethanesulfonyloxyoctane, some ten times less active than Myleran itself caused similar effects rather more slowly (Fig. 2, Galton, 1953b) and only after repeated daily doses for as long as 2 weeks had been given.

Fig. 1. Chronic myeloid leukemia. Case 7. Male, 40 years Blood counts showing effects of excessive doses of Myleran. Note severe neutropenia in the fifth month and depression of hemoglobin level from the sixth month after the beginning of treatment

In chronic myeloid leukemia, the leukemic myeloid tissue is rather more sensitive than its normal counterpart, as may be seen by comparing the percentage depression of mature neutrophils in leukemic and nonleukemic subjects following a single large dose of Myleran. Doses of 2-3 mg per kilogram given in 1-5 days to patients with chronic myeloid leukemia were often strikingly and rapidly effective, and were sometimes well tolerated; but in 3 cases profound bone marrow depression resulted (Fig. 1) which proved fatal in one case (Fig. 2; see also case 1, Bollag,
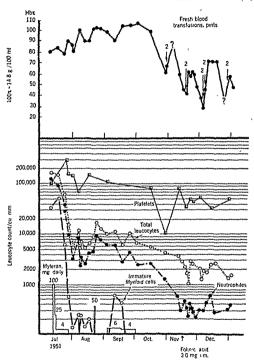
FIG 2. Chronic myeloid leukemia. Case 13 Female, 47 years. Blood counts showing effects of excessive doses of Myleran. Note delayed and progressive depression of hemoglobin, platelets, and neutrophiles Terminally the bone marrow was hypocellular and myeloblastic.

1953b). In other cases the use of large doses was associated with the rapid onset of relapse which proved to be resistant to Myleran. Large doses, therefore, are dangerous, and often ineffective.

A standard daily dosage of 0 06 mg. per kilogram of body weight (about 4 mg daily) has proved to be satisfactory. Symptomatic relief and the rate of hemoglobin increase are as rapid at this dosage as with the large doses The rates of splenic regression and of the fall in leucocyte count are slower but the spleen regresses quickly enough for pain to be relieved and a slowly falling leucocyte count is an advantage in the subsequent management of the case, should maintenance therapy be required. Franzen (1954) has used radiotherapy to the spleen in combination with Myleran, to reduce the size of the spleen rapidly This is necessary to reduce the period of in-patient treatment for patients living in remote parts of Sweden. Franzen finds that very small doses of x-rays are then effective. Kurrle (1955) found that splenic regression following Myleran therapy was no slower than after radiotherapy.

Myleran may be given in separate courses, or continuously. At standard dosage, a course lasts up to 7 months. For continuous therapy, the daily dose requirement varies from time to time according to the trend of the leucocyte count The smallest maintenance dose that has been used is 0 5 mg daily. Most patients require 1–3 mg. daily. Some need to continue taking their starting dose. The leucocyte count begins to rise within a few days of discontinuing the drug, even when administration has been continuous for months or years. If the daily dose is raised or lowered by 0.5 mg. significant alterations in the trend of the leucocyte count are produced. Myleran is not, therefore, a cumulative drug.

The absence of gastrointestinal upset, even following administration of large doses, has already been mentioned. In this alone, Myleran has great advantage over most other agents in use. The only side effects observed in patients receiving Myleran have been pigmentation of the skin in several subjects, permanent amenorrhoea in premenopausal women (Bollag, 1953b; Galton, 1953a), and mild transient gynecomastia in 2 men. At least one healthy infant has been born to a mother treated with Myleran during pregnancy (Dameshek, 1953a), but in view of the possibility of adverse genetic effects on the fetus, Myleran should probably not be given in pregnancy, except after termination.

2. The Untreated Patient

When a patient with chronic myeloid leukemia is first seen, it is difficult to predict the course of his disease from the clinical and hematological findings. Patients with apparently chronic disease may follow an acute course whereas those thought to be gravely ill who are treated

in the same way may respond extremely well. Case 22 (Fig. 3) is an example of unexpectedly good response in a very sick man. Case 23 (Fig 4) is an example of smooth response to treatment over a 2-year period in a patient at an early stage of chronic myeloid leukemia. Similar cases have been described elsewhere (Galton and Till, 1955; Wintrobe *et al.*, 1954; Hansen, 1954; Bollag, 1953b; Galton, 1953a,b).

Several points of contrast between cases 22 and 23 are to be noted:

(1) In both cases treatment was stopped when the leucocyte count had reached reasonably normal levels. By this time both patients were
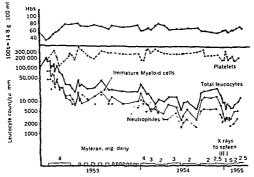
Fig. 3 Chronic myeloid leukemia Case 22. Male, 36 years No previous treatment. Continuous Myleran therapy was necessary for control. Blood counts showing persistance of high immature myeloid cell count. Two years later received deep x-rays to the grossly enlarged spleen.

symptomatically improved, had rising hemoglobin levels, regressing spleens But in case 23 the numbers of immature leucocytes in the blood had fallen to very low levels, whereas in case 22 they remained high

(2) In the month after stopping treatment the leucocyte count was almost unchanged in case 23 but had risen to 45,000 per cu. mm. in case 22 In case 23 the patient remained symptom-free without further treatment for 14 months, whereas the other patient has required maintenance therapy throughout

(3) The spleen in case 23 regressed during treatment, became impalpable, and began to enlarge slowly during the next year. The spleen in

case 22 after initial dramatic regression enlarged again in spite of maintenance therapy. Two years later this enlargement gave rise to pain, which was relieved by small doses of radiotherapy given in addition to Myleran. Only slight regression of the spleen followed. The difference in response and ease of control in these two patients probably reflect differences in the stage and activity of the disease. Many patients treated with Myleran for the first time, whether they have received other treatment previously or not, respond to the drug as in case 23. They can safely be left without further treatment until evidence of relapse appears. Their
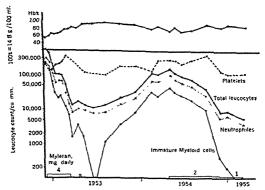
Fig. 4 Chronic myeloid leukemia. Case 23. Male, 47 years. No previous treatment. Excellent control by 2 courses of Myleran in 2 years. Blood counts showing hemoglobin level well maintained

response to subsequent courses of Myleran will probably be as satisfactory as the first but relapse is likely to appear sooner, and these patients will eventually require continuous therapy if they are to be maintained in remission. Patients with more active disease or in its later stages are more likely to respond as in case 22; they tend to relapse quickly when treatment is withheld and require continuous therapy from the beginning.

3. Patients Previously Treated

The response to Myleran of patients already treated by x-rays or other methods again depends on the stage of the disease. (a) The patients

may still be responding well. (b) They may have responded well initially, and later ceased to do so. (c) They may have responded well but relapsed quickly. (d) They may have failed to respond from the beginning. It is of course necessary to be sure that adequate treatment has been given; treatment is often said to have failed, when it has not been given a fair trial, having been abandoned with insufficient justification. (e) They may be in myeloblastic relapse.

(a) If the patient is still responding well, and likely to do so again, he is equally likely to respond to Myleran and may react like a patient who has not received any treatment. Case 21 (Fig. 5) is a patient who responded well to urethane for 1 year, would probably have done so again,
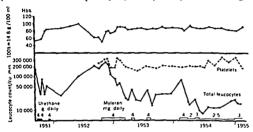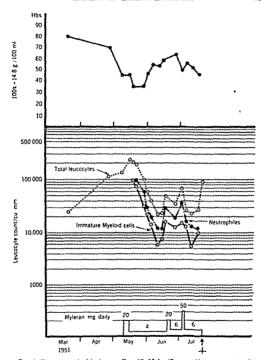
FIG. 5 Chronic myeloid leukemia. Case 21. Female, 38 years. Blood counts showing response to Myleran therapy after treatment with urethane. After one course of Myleran, maintenance therapy was given.

but was given a course of Myleran instead because of its freedom from side effects. She obtained an excellent remission, was left untreated for 1 year, and was subsequently well controlled by maintenance therapy which she has had for 18 months. Another patient developed priapism 9 days after starting his second course of radiotherapy to the spleen. Owing to the pain provoked by moving him, radiotherapy was abandoned in favor of Myleran to which he responded as well as he probably would have done to x-rays. A young married woman lived so far from a radiotherapy center that treatment necessitated admission. To enable her to remain in her home town, where there are excellent hematological facilities but none for radiotherapy, she was given continuous Myleran therapy when she relapsed, and has been in excellent health for 2 years. In these cases Myleran was substituted for urethane or radiotherapy for reasons of convenience only.

(b) With conventional radiotherapy to the spleen it is usual for remissions to become progressively shorter so that the intervals between courses of treatment lessen. Sometimes, however, long remissions unexpectedly occur after a period of supposedly lessening response to treatment (Ledlie, 1953). If a new form of therapy is given in such circumstances, the ensuing long remission might be attributed to the superiority of the new treatment, when in fact fluctuation in the activity of the disease process was responsible. In the case of Myleran, long remissions have occurred frequently after progressively shortening x-ray-induced remissions and are unlikely to reflect change in the disease process. (Galton, 1953a; Galton and Till, 1955; Kurrle, 1955). It is possible, however, that equally good remissions might have followed alternative forms of radiotherapy, such as whole-body irradiation or P^{32}. But it is to be noted that the immediate response to splenic irradiation in these patients has usually been satisfactory as evidenced by symptomatic relief, splenic regression, hemoglobin rise, and rate of fall of leucocyte count. In each case it has been the speed of relapse that lessened the value of radiotherapy and led to the decision to use Myleran instead.

(c) Sometimes patients relapse rapidly even after the first course of radiotherapy. If they respond no better to a second course, radiotherapy should be abandoned. In such cases the disease may sometimes be controlled for long periods by means of Myleran.

Patients relapsing rapidly always require continuous Myleran therapy. Attempts to induce remission by means of large doses are likely to lead not only to rapid relapse but also to Myleran-resistance (Fig. 8, Galton, 1953b). But, when standard doses are given, the steep rise in leucocyte count is arrested usually within 6 weeks, followed by a slow steady fall, taking as long as 1 year before normal levels are reached. Even greatly enlarged spleens may become impalpable during this period, as in a patient who had previously received 11 courses of radiotherapy to the spleen in 8 years (case 4, Galton, 1953a). The hemoglobin level may begin to rise soon after treatment is begun but sometimes continues to fall for as long as 4 months before rising (case 29, Galton and Till, 1955); in spite of this, relief of symptoms is almost always evident during the first month of Myleran therapy and treatment may be continued without increasing the dose of Myleran. The hemoglobin level may sometimes rise, or cease to fall, although the effect on the leucocyte count is comparatively slight. One patient had required blood transfusions every 6 weeks for more than a year when Myleran therapy was begun, after which his hemoglobin level rose spontaneously to 85% (12.6 g. per 100 ml.) and was kept there for 18 months. But the leucocyte count never fell below 50,000 per cu. mm. This man's leucocyte count rose slowly from

FIG 6 Chronic myeloid leukemia. Case 10. Male, 47 years. No response to radio-therapy (completed February 23, 1951). Blood counts showing poor response to Myleran Large doses were given.

the time the dose of Myleran was reduced to 2 mg. daily. He might have fared even better than he did if a slightly higher dose had been given.

(d) Patients referred for Myleran therapy are sometimes described as "radio-resistant." Often these prove to have received inadequate therapy, treatment having been abandoned as ineffective because improvement
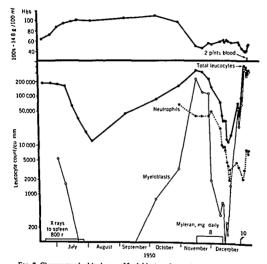
FIG 7   Chronic myeloid leukemia   Myeloblastic relapse. Case 2. Female, 58 years. Previously treated with radiotherapy, urethane, and radioactive phosphorus. Blood counts showing dramatic but fleeting response to Myleran.

had not occurred quickly enough. In the only case in the writer's experience in which radiotherapy had been pushed to its limits without producing any benefit, Myleran was of little value (case 10, Fig. 6), though the rapidity of relapse after the initial response was probably a result of the type of dosage employed.

(e) Patients in acute myeloblastic relapse derive no real benefit from

Myleran even though they have never been treated previously with it; but the leucocyte count may fall rapidly and the spleen may regress enough to relieve pressure symptoms. When the myeloblast count rises quickly, fever and prostration are prominent symptoms. As the count falls under the influence of treatment these symptoms vanish, only to return as the count rises again (case 2, Fig. 7). In the case quoted the hemoglobin fell rapidly as the myeloblast count rose: the fall was arrested during the period of Myleran therapy but continued when the myeloblast count rose again.

Patients in myeloblastic relapse are better treated with deacetyl-methylcolchicine (Moeschlin *et al.*, 1954) or 6-mercaptopurine (see below).

4. Treatment of Patients Resistant to Myleran

Myleran resistance appears in two ways: (1) acutely, (2) insidiously.

A. *Acute Myleran Resistance.* This has developed in the first few months of Myleran therapy and has always been associated with the use of intermittent dosage or when a course of treatment has been terminated too soon, or when inadequate maintenance dosage has been given; it has shown itself by a rise in the immature cell count which continues in spite of resumed treatment even with excessively large doses. In one case crops of leukemic skin nodules appeared. If Myleran therapy is continued even when the resistant myeloid cell population is increasing, the adult neutrophil count and platelet count will fall and agranulocytic angina and purpura may supervene.

In case 27 (Fig. 8) the initial course of Myleran was discontinued too soon, and when administration was resumed the leucocyte response was poor and terminal relapse soon followed.

B. *Insidious Myleran-Resistance.* This has appeared in patients treated for long periods by maintenance therapy. It has shown itself in two ways, first by increase in the numbers of immature leucocytes, particularly myeloblasts; secondly, by insidious enlargement of the spleen, the hematological and clinical states remaining satisfactory. The maintenance dose if increased fails to check enlargement of the spleen or the rise in the absolute immature leucocyte count. If treatment is stopped, terminal relapse follows rapidly.

C. *Treatment.* In the acutely developing type of Myleran resistance, 6-mercaptopurine or methylcolchicine might be of temporary value, but these drugs were not available when the condition appeared in the writer's patients

In one case myeloblastosis appeared during a period of apparently satisfactory clinical and hematological control 4 years after onset of the

disease (see Fig. 2, Galton and Till, 1955). The spleen was only moder-
ately enlarged. Myleran therapy was stopped and 6-mercaptopurine
was given. This caused regression of the spleen and controlled the leuco-
cyte count but the hemoglobin level began to fall rapidly. Transfused
blood was rapidly destroyed and the patient became jaundiced after
transfusion. Although the direct antiglobulin test was negative, cortisone
was given. Subsequent transfusions did not give rise to jaundice but the
hemoglobin level fell nearly as quickly as before. ACTH gave no better
result than cortisone. Splenectomy was only slightly more effective. The
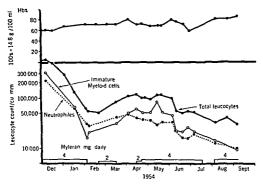
Fig 8 Chronic myeloid leukemia Case 27. Female, 63 years. Blood counts showing
resistance to second course of Myleran following premature termination of first course.

myeloblast count was kept below 500 per cu. mm. by 75–150 mg. daily
of 6-mercaptopurine The patient remained in remarkably good general
condition for 6 months after the onset of myeloblastosis, and this may be
attributed to the effect of 6-mercaptopurine in keeping the myeloblast
count at a low level.

Case 20 (Fig. 9) is somewhat similar to the last but the rate of hemo-
globin fall was slower and a satisfactory response was obtained with
6-mercaptopurine alone. The patient continued working throughout this
period, apart from 2 days in hospital for transfusion.

In case 18 (Fig. 10) Myleran-resistance appeared as slowly progressive
splenomegaly. The patient had been well controlled by Myleran for 2
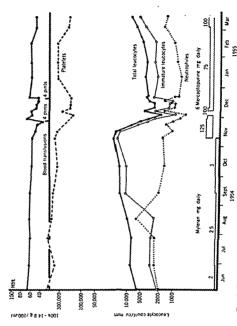
Fig. 9. Chronic myeloid leukemia. Case 20. Female, 47 years. Blood counts showing onset of myeloblastic relapse during maintenance therapy with Myleran and temporary response to 6-mercaptopurine. Previously well controlled for 1 year with nitrogen mustard and for 2 years with Myleran.

years; the hemoglobin, leucocyte, and platelet counts were relatively
stable but over a period of 6 months the spleen enlarged, eventually
extending below the pubis and to the right anterior superior iliac spine.
Myleran administration was stopped to permit radiotherapy to be given
to the spleen; pain was relieved but only slight regression ensued, and the
hemoglobin began to fall rapidly from the day Myleran therapy was
stopped Two blood transfusions were given but myeloblastosis followed
and was not influenced by 1 week of treatment with Myleran which was
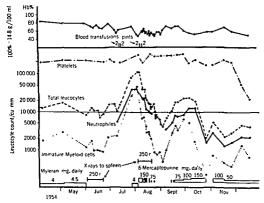
Fig 10 Chronic myeloid leukemia. Case 18. Male, 33 years Well controlled for
2 years with Myleran Gross splenomegaly during maintenance therapy Blood counts
showing poor response to radiotherapy and temporary response to 6-mercaptopurine

therefore abandoned Treatment with 6-mercaptopurine was begun con-
currently with further radiotherapy to the spleen which had again become
enormous. Considerable splenic regression took place with relief of pain
and the leucocyte count fell and spontaneous rise of hemoglobin followed.
The benefit was shortlived but the patient continued his work until
10 days before he died 4 months later.

Myeloblastosis seems to occur frequently when Myleran is withheld
after a long period of maintenance therapy. Therefore, in case 22 (Fig. 3),
which was similar to that just described, x-rays were given to the spleen

during a period of reduced Myleran dosage. Whether the response was better because of this or simply because the patient had not yet entered the unstable phase of disease cannot be said. Bollag's patient (1954b) responded for several months to radiotherapy to the spleen after Myleran had been discontinued. A good remission was then obtained with methylcolchicine but the patient quickly relapsed when treated again with Myleran, though he still proved to be sensitive to methylcolchicine.

Whatever form of treatment is used initially, resistance to it eventually develops. Subsequent response may still be obtained with one or more of the other methods. Myleran has proved of value when resistance has developed to x-rays, urethane, nitrogen mustard, 6-mercaptopurine (Burchenal, 1954), and 1,3-bis(ethyleneiminosulfonyl)propane. 6-Mercaptopurine and deacetylmethylcolchicine (Bollag, 1954b) have been effective after the onset of resistance to Myleran. In the acute leukemias successive therapy with three classes of drugs, corticotropin and the steroid hormones, the folic acid antagonists, and the purine antagonists, has led to significant prolongation of survival (Tivey, 1954). It is possible that a similar prolongation may yet be achieved in chronic myeloid leukemia by the appropriate use of the several methods now available.

5. The Place of Myleran in Routine Management

In this article, considerable emphasis has been placed on the unsatisfactory aspects of Myleran therapy. In some of the instances quoted, the poor response was a result of faulty handling of Myleran, in others the intrinsic deficiency of the drug was the reason for its failure. Nevertheless it is a most useful drug. It is chemically stable, is readily absorbed from the gastrointestinal tract, and is almost free from side effects. Its effects on the leucocyte count are remarkably uniform, so that in chronic myeloid leukemia the fall can be adjusted with precision to the desired rate by making appropriate changes in dosage (Table II, Galton and Till, 1955). Some patients have been maintained on the same dose for over 2 years. Provided large doses are avoided and rigid hematological control is practiced, Myleran is a safe drug. The minimum requirements for routine management have been stated elsewhere (Galton and Till, 1955) and the advantages and disadvantages of the "course" and "continuous" methods of treatment were also discussed; these matters need no repetition. Here an attempt is made to define the place of Myleran amongst the numerous other available palliatives. In its efficacy and the precision of control afforded by it, Myleran compares favorably with all other available methods. It is inexpensive, its administration requires no specialized personnel or equipment and therefore remains in the hands of the physician. The freedom from side effects is shared only by radio-

active phosphorus. Nevertheless it has been used for only 6 years, compared with 20 years for radioactive phosphorus and over 50 years for external radiotherapy. It is probable that methods of using Myleran will be improved. Meanwhile it would be invalid to attempt a statistical analysis of survival by Boag's method (Tivey, 1954), since in many of the patients so far treated, the drug has almost certainly been wrongly used. But, if Myleran correctly used alone should prove to lead more frequently to earlier terminal relapse than radiotherapy (Blackburn et al., 1956), then the drug will be contra-indicated as the first line of treatment. As long as treatment remains palliative, and resistance develops to each method, management of each patient will involve the use of several agents in succession. In general, radiotherapy is still the treatment of choice, either as external x-irradiation to the spleen, as total-body irradiation, or as oral or intravenous radioactive phosphorus. Myleran should be used first if radiotherapy facilities are not available, or if travel to and from a radiotherapy unit involves hardship for the patient, either because of his condition or because of loss of working time. If Myleran is used first, it is probably best to give a single course lasting 6 or 7 months and then leave the patient without treatment as long as he is free of symptoms. When relapse ensues he may then require maintenance therapy. At most clinics, however, radiotherapy is available and repeated courses are given. At some point relapse to treatment lessens, or relapse comes quickly. In some cases response is unsatisfactory from the beginning, or the first relapse appears within a few months of treatment. At this stage the method of irradiation may be changed, or chemotherapy is begun. There is no longer any justification for starting with drugs such as benzene, arsenic, or urethane that cause side effects. Triethylene-melamine is favored by some but is less regularly effective than Myleran, control is more difficult, the dose requirements are dangerously variable, and some patients require large doses (Bond et al., 1953) and may develop anorexia and nausea. The risks of bone marrow damage are considerable even with the greatest care (Meyer et al., 1952; Blackburn and King, 1954; Wintrobe et al., 1954). Myleran is probably the drug of choice and patients treated with it may remain in good health for several years before they become resistant. In most patients no longer responding to radiotherapy, maintenance therapy will be required. It is difficult to define the stage at which Myleran therapy should be given up and another drug used. In some of the cases reported in this article, Myleran therapy may have been continued too long. It may be better to change to 6-mercaptopurine or to methylcolchicine or to 1,3-bis(ethyleneiminosulfonyl)-propane as soon as the patient on maintenance therapy with Myleran shows any evidence of becoming resistant, such as increase in the size of

the spleen, or increase in the absolute numbers of myeloblasts. In this way, another respite may be gained. But the patient inevitably enters a terminal phase resistant to all treatment.

V. THE ACTION OF MYLERAN IN CHRONIC LYMPHATIC LEUKEMIA, IN ACUTE LEUKEMIAS, AND IN OTHER CONDITIONS

Leukemic lymphocytes are more sensitive to the effects of ionizing radiations and to drugs such as urethane, nitrogen mustard, and triethylenemelamine than are normal lymphocytes. In spite of the relative
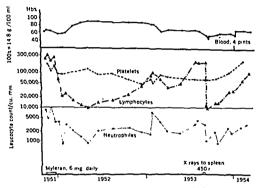
FIG 11. Chronic lymphatic leukemia. Female, 63 years. Blood counts showing good response to Myleran. Note temporary depression of platelet count at end of course

insensitivity of normal lymphocytes to Myleran, a trial of lymphatic leukemia seemed indicated. Figure 12 shows the effect of Myleran on the leucocyte count (90% lymphoblasts) in a young man with acute lymphoblastic leukemia. Considerable fall in the leucocyte count occurred, and some regression of lymph nodes, liver, and spleen occurred, but no remission followed. In the first case of chronic lymphatic leukemia treatment resulted in complete regression of enlarged lymph nodes, fall in leucocyte count, and more significant, sustained rise in hemoglobin level. No significant regression in the size of liver and spleen took place; nor did
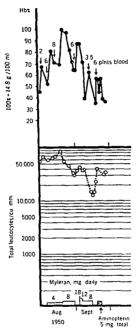
Fig. 12 Acute lymphoblastic leukemia Male, 20 years Blood counts showing effect of Myleran on leucocyte count No remission was obtained

these organs regress when radiotherapy and radioactive phosphorus were used 2 years later (Fig. 11).

Two other patients were subsequently treated. In both the lymphocytes proved to be very sensitive to Myleran, and lymph nodes, in one case much enlarged, regressed almost completely but the size of the livers and spleens was unaffected In 2 of the 3 cases the platelet counts were
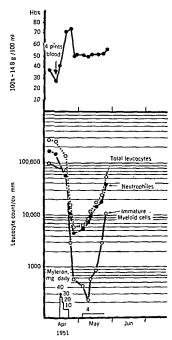
Fig. 13. Subacute myeloid leukemia. Case 9. Female, 60 years. Blood counts showing fleeting response to large doses of Myleran and failure of attempted maintenance with standard dosage.

depressed below 50,000 per cu. mm. six weeks after treatment was begun, and clinical purpura appeared. This is a recognized hazard with chronic lymphatic leukemia and may occur with any other form of treatment. Thus cautious trial of Myleran is warranted in chronic lymphatic leukemia.

Myleran has not been useful in acute leukemias; several patients with myeloblastic leukemia and one with monocytic leukemia have been treated; the leucocyte counts fell and the differential counts showed some improvement, but in no instance was remission obtained. The platelet counts were low before treatment and were depressed further by it. Dameshek (1953a) obtained a remission in a pregnant woman suffering from monocytic leukemia. As monocytic leukemia tends to be refractory to the forms of therapy usually employed in acute leukemia, Myleran may therefore be tried when other methods have failed. Dameshek (1953b) has also reported Myleran to be of value in some cases of subacute myeloid leukemia. This is an ill-defined clinical entity which in some respects is intermediate between acute and chronic myeloid leukemia. In case 9, Myleran produced a dramatic effect on the patient's well being, on the size of the liver and lymph nodes, and on the leucocyte count, but the thrombocytopenia did not improve, and no spontaneous increase in hemoglobin took place (Fig. 13).

In one patient with myelofibrosis, Myleran caused regression in size of liver and spleen, fall in the leucocyte count, and disappearance of primitive cells from the blood. But the hemoglobin level continued to fall, and this fall became more rapid with the onset of purpura and hemorrhages from mucous membranes resulting from thrombocytopenia. Myleran has not been used in polycythemia vera. It has induced remission in one case of Hodgkin's disease (Burchenal, 1954) but was ineffective in one patient with terminal disease treated by the writer. It is probable that the limiting factor will always be the extreme vulnerability of the myeloid and megakaryocytic tissues. The megakaryocytes are, however, more resistant than the myeloid cells and during the first few months of treatment in chronic myeloid leukemia, the platelet count often rises, sometimes to high values (Kurrle, 1955). The bone marrow of patients with chronic myeloid leukemia who have received maintenance therapy for many months has several times shown marked relative increase in the numbers of megakaryocytes in an otherwise somewhat hypocellular marrow. Rider (1954) has obtained remission in a patient with leukemic thrombocythaemia; the megakaryocytes were more sensitive to Myleran than in most cases of myeloid leukemia. Hansen (1954) reported relief of pain in a patient with multiple myelomatosis and further trials would be desirable.

VI. Conclusions

In this article, the writer has attempted to discuss some of the problems of clinical assessment in the chronic leukemias Clinical studies with Myleran, one of the newer chemotherapeutic agents, have been chosen to illustrate them. The same problems are likely to arise with each new remedy submitted for clinical trial until a radical form of therapy is found, when the clinical effects will probably become quickly apparent. Patients suffering from leukemia show great variation in mode of onset, severity of disease, rapidity of progression, response to treatment. Even the diagnostic criteria of leukemia are not clear. A series of cases could easily be assembled showing at one end transition forms between typical chronic myeloid leukemia and typical acute myeloid leukemia; and at the other end transitional forms between chronic myeloid leukemia and other myeloproliferative disorders such as myelosclerosis or polycythemia vera would be found (Black-Schaffer and Stoddard, 1953; Hutt et al., 1953). Similar transition forms link chronic lymphatic leukemia to diffuse lymphosarcoma and lymphosarcoma of focal origin. As long as our nomenclature in these maladies remains only descriptive, it is impossible to know whether the extreme variants represent widely differing manifestations of a single disease process, or whether the transition forms link etiologically distinct diseases only by superficial similarities

One danger in collecting large series of cases for statistical purposes is that the diagnoses on the case sheets have to be accepted as they are. Yet the diagnostic criteria of different physicians and pathologists vary greatly, and in analyzing the records of deceased patients, essential information that might necessitate revision of the stated diagnosis is often lacking. Patients classified as subleukemic lymphatic leukemia at one hospital may appear under lymphosarcoma at another. Series of cases of lymphatic leukemia assembled independently at the two hospitals might suggest that treatment was better at one of them, whereas the difference would in fact reflect only the different composition of the series.

Each patient with leukemia presents a different problem in treatment which requires individual attention. It is therefore very difficult in practice to arrange a comparative trial of two methods of treatment by random sampling There is no justification for withholding a remedy known to be of value in order to try a new form of treatment. This means that the first trials of a new remedy are always carried out on patients in whom conventional remedies have ceased to be of value. In chronic myeloid leukemia we have already seen that resistance tends to develop to whatever form of treatment is given. The fact that a subsequent response may be obtained with a different remedy does not necessarily

mean that this treatment is better than the first: only that the disease is still in a responsive stage.

The research worker must often be impatient at the clinician's reluctance to report on the value of a new remedy he has been using for a year or more The reason for the clinician's attitude is that the immediate results of treatment are not of great significance. Many agents now available will bring about quite rapid clinical and hematological improvement; but it is the long term results that matter to the patient to whom a spectacular remission followed a few months later by relapse is of no real value. Preliminary reports of most new remedies are almost always over optimistic: as the trial proceeds the numbers of therapeutic failures increase, previously unsuspected side-effects or delayed toxic manifestations appear, and the most successful cases often prove to be very chronic examples in which similar results might have been obtained with any other available treatments. Several years must elapse before a reasonable statement can be made about the value of a remedy, or detailed recommendations given regarding its place in the overall management of the patient. With the group of remedies now available it is a matter of degree that makes one better than another; such considerations as ease of administration, frequency of side effects, extent and regularity of response, efficacy in maintenance, safety are all important. Judged by these standards, Myleran, in its limited field of application ranks highly, but it is probable that if the survival of the chronic leukemias is increased in the near future, it will be a result of "the judicious use of the whole therapeutic armamentarium that is available" (Wintrobe et al , 1954).

VII. Case Reports

Case 23 (Fig. 4). A man aged 47 years noticed weakness in October, 1952, and shortly after complained of pain in the left hypochondrium when lifting. He had lost his appetite, could not tolerate fatty food, had fluid regurgitation, and since August, 1952 had lost 14 pounds weight. He was first seen on December 1, 1952 and was found to have an enlarged spleen 14 cm. below the left costal margin and extending to the midline. A blood count showed Hb 56% (8.3 g per 100 ml.), leucocytes 500,000 per cu. mm. (190,000 immature myeloid cells).

Treatment and progress. Myleran 4 mg. daily was given from December 5, 1952 to March 13, 1953 (total 396 mg), and again from April 17 to 24, 1953. His condition improved rapidly, the spleen regressed until it could not be felt (June 22, 1953) and the Hb level increased steadily to 91% (13 9 g. per 100 ml.) on August 7, 1953 He remained well until April, 1954 when he again complained of tiredness, loss of weight, and of indigestion.

The spleen was easily palpable. A blood count (April 23, 1954) showed Hb 74% (11.0 g. per 100 ml.), leucocytes 150,000 per cu. mm. (46,000 immature myeloid cells), platelets 280,000. Myleran 2 mg. daily was given until November 26 and 1 mg. daily until March 4, 1955 when he was fit, the spleen was not felt and Hb was 87% (12.9 g. per 100 ml.), leucocytes 5,600 per cu. mm., no immature cells seen. He was fit when last seen in January, 1956.

Case 21 (Fig. 5). Housewife aged 38 years. In 1950, following a normal delivery, she complained of headaches, anorexia, and lassitude, and noticed that her abdomen was increasing in size. Her periods were frequent, irregular and the loss was excessive. In September, 1951 she was found to be thin and flushed. The spleen occupied most of the left side of the abdomen and the liver was just felt. Rubbery nodes were felt in the axillae. Chronic myeloid leukemia was diagnosed and she was treated with urethane, 4 mg. daily. Her symptoms were relieved, the spleen regressed moderately. Her periods became regular. In October, 1952 she was again anemic and complained of abdominal discomfort. The spleen was 24 cm. below the left costal margin and 6 cm. to the right of the midline.

Treatment and progress. Myleran 4 mg. daily was given from October 27, 1952 to March 11, 1953, (total 536 mg. in 134 days) and 4 mg. daily from April 28, to May 12, 1953 and from July 14, to August 11, 1953. Symptoms were rapidly relieved and the spleen regressed. On March 10, 1953 it was 6 cm. below the left costal margin and 6 cm. to the left of the midline. In January, 1954 symptoms had returned, the spleen was again enlarged, and Myleran was given as follows:

> 4 mg. daily from February 2–March 16, 1954
> 2 mg. daily from March 16–April 27, 1954
> 3 mg. daily from April 27–May 25, 1954
> 2 5 mg. daily from May 25–December 14, 1954
> 3 mg. daily from December 14, 1954–May 30, 1955.

Her condition was excellent throughout this period and after the first course of Myleran the hemoglobin level was maintained between 83 and 97% (12.3 and 14.4 g. per 100 ml.). From June 1955 to August 1955 the haemoglobin level fell from 83% to 34% (12 3 to 5 g. per 100 ml.) and the myeloblast count rose from 0 to 2000 per cu. mm. 6-mercaptopurine therapy was ineffective and she died on September 24, 1955, the myeloblast count having reached 55,000 per cu. mm. two days before death.

Case 10 (Fig. 6). A man of 47 years was admitted on May 8, 1951 complaining of tightness and fullness in the abdomen after meals and of increasing lassitude and tiredness for 2 months. Chronic myeloid leukemia

had been discovered accidentally 7 months before and radiotherapy to the spleen had been given elsewhere without effect (2700 r. in 11 weeks completed February 23, 1951). On examination the spleen was firm and tender, its lower pole was felt 21 cm. below the left costal margin and the right border in the midline. There were no other relevant physical signs. On admission Hb was 54% (8.0 g. per 100 ml.), W.B.C. 134,000 per cu. mm., immature myeloid cells 54,270 per cu. mm. of which 11,400 were myeloblasts. Six days later the leucocyte count was 231,000 per cu. mm , immature myeloid cells 132,590 per cu. mm., of which 11,200 were myeloblasts.

Treatment and progress. Myleran was given as follows: 20 mg. daily from May 15–19; 4 mg. daily from May 20 to June 19; 20 mg. daily from June 20–22; 6 mg. daily from June 23 to July 2; 50 mg. on July 3 and 4; 6 mg. daily from July 5 to 20 (total 530 mg. in 66 days). His general condition improved during the first 6 weeks of treatment; the spleen regressed slightly but remained hard. On May 26 enlarged cervical nodes were felt; they increased rapidly in size and number 1 month later when similar nodes appeared in both axillae, iliac, and inguinal regions. Following the very large doses of Myleran given on July 3 and 4 the nodes regressed slightly but the spleen increased rapidly in size and he died on July 21, 1951 after a few days of unexplained pyrexia The blood count on June 11 showed Hb 59% (8.8 g. per 100 ml.), W B.C. 21,700 per cu. mm., immature myeloid cells 6250 per cu mm., of which 540 were myeloblasts. On June 27, Hb was 69% (10.2 g. per 100 ml.), W.B.C 34,500 per cu. mm., immature myeloid cells 12,075 per cu. mm., of which 1725 were myeloblasts

Case 2 (Fig. 7). Widow aged 58 years. Chronic myeloid leukemia was diagnosed in December 1948, symptoms having been present for more than 1 year. She was treated by radiotherapy to the spleen, 220 kv. 500 r. in 27 days with improvement. In March, 1949 symptoms recurred and she was given urethane, 3 g. daily for 6 weeks This was discontinued because of nausea; a further course of radiotherapy to the spleen was given By February, 1950 she was again weak and the spleen was grossly enlarged. 2.8 mc. of P^{32} was given intravenously on March 1, 1950 and another course of radiotherapy to the spleen was given in July 1950. She remained well until November, 1950 when she developed headaches, severe indigestion, nausea, vomiting, rapid swelling of the abdomen with pain in the left side. The spleen almost filled the abdomen and a blood count on November 15, 1950 showed Hb 50% (7.4 g. per 100 ml), W.B.C. 337,000 per cu. mm., 286,450 immature myeloid cells, of which 220,000 were myeloblasts.

Treatment and progress. Myleran 8 mg. daily was given from November 16, to December 11, 1950 (total 208 mg). Symptoms were relieved, she regained her appetite and the spleen regressed 5 cm. medially and upwards On December 15, 1950, Hb was 61% (9 g. per 100 ml.), W.B.C. 31,300 per cu. mm., 19,000 immature myeloid cells none of which were myeloblasts. On December 22, 1950 the spleen was harder and as large as before treatment was begun and 5 days later she was feeling ill, anoretic, and complained again of abdominal pain. Hb was 56% (8.3 g. per 100 ml), W B C. 74,500 per cu. mm., 6900 immature myeloid cells, of which 5500 were micromyeloblasts. Myleran therapy was resumed on December 29, but she became rapidly worse, purpura followed and she died from extensive cerebral hemorrhage on January 8, 1951.

Case 27 (Fig. 8). A widow aged 63 years was admitted on November 27, 1953 in congestive cardiac failure. She complained of progressive weakness and loss of weight for about 1 year and of dyspnea, swelling of the ankles and abdomen for 3 months. On examination she was dyspneic, was found to have tachycardia, enlargement of the heart, edema of legs and back, and bilateral pleural effusions. The liver and spleen were enlarged and the liver was tender. A blood count showed Hb 54% (8 g. per 100 ml.), W.B.C. 651,000 per cu. mm., 365,000 immature myeloid cells, of which 28,000 were myeloblasts.

Treatment and progress. Myleran 4 mg. daily was given from November 29, 1953 to February 2, 1954. Improvement was dramatic. Signs of congestive failure disappeared, spleen and liver regressed. On February 2, 1954, Hb was 70% (10 4 g. per 100 ml.), W.B.C. 62,200 per cu. mm., 16,000 immature myeloid cells, of which 620 were myeloblasts. Myleran 2 mg. daily was given from February 24 to March 9, 1954 and from April 5-15. The dose was then increased to 4 mg daily until July 16, 1954 4 mg daily were given from August 6 to September 17 From March to June, 1954 the blood count remained substantially unchanged. On June 3, Hb was 78% (11.5 g. per 100 ml.), W.B C. 94,400 per cu. mm., 44,400 immature myeloid cells, of which 6600 were myeloblasts. On September 3, the spleen was smaller than at any time previously and measured 6 cm. below the costal margin and 4 cm to the left of the midline. Hb 83% (12 3 g. per 100 ml.) W.B.C. 27,000 per cu. mm., 9300 immature myeloid cells of which 270 were myeloblasts. One month later, she complained of pain in the left hypochondrium and of dyspnea. She was readmitted. On examination, she was dyspneic, orthopneic, febrile. The tender spleen was 15 cm. below the costal margin and reached the midline, the liver was 10 cm. below the costal margin Hb was 77% (11.4 g. per 100 ml.), W B.C. 121,700 cu. mm , 48,600 immature myeloid

cells. Myleran 4 mg. daily was given from October 1, 1954, but she failed to respond and she died on November 4, 1954.

Case 20 (Fig. 9). Female, 47 years first noticed swelling of ankles and complained of lassitude and malaise in December 1950. In August, 1951 a diagnosis of chronic myeloid leukemia was made and she was treated elsewhere with nitrogen mustard. Her condition improved and she returned to work. Further nitrogen mustard was given at intervals until September, 1952 when a course of Myleran was given. She received no further treatment until February, 1954 when the spleen was again considerably enlarged (15 cm. below the costal margin and 3 cm. to the right of the midline), Hb 56% (8 3 g. per 100 ml.), W.B.C. 128,000 per cu. mm. She was treated again with Myleran and maintenance doses of 2–3 mg daily were required. Only moderate splenic regression occurred and the spleen was never less than 6 cm. below the costal margin and 2.5 cm. to the left of the midline. Hb level reached 76% (11 2 g. per 100 ml.) in March 1954, but from June onwards never exceeded 60% (8.9 g. per 100 ml.). Frank myeloblastosis appeared in September and on November 9, 1954 Myleran administration was discontinued and 6-mercaptopurine 125 mg. daily was begun. The Hb level fell rapidly and 2 blood transfusions of 4 pints each were given on November 27 and December 21, 1954. The subsequent fall in Hb level was much slower (2.5 g. per 100 ml. in 82 days). The leucocyte count was kept below 10,000 per cu. mm. although the differential count improved little. The spleen regressed considerably and on January 25, 1955 was 4 cm. below the costal margin and 4 cm. to the left of the midline. The patient continued her work until a few days before she died in acute relapse in April 1955.

(Fig. 11). Chronic lymphatic leukemia. A woman of 63 years had had two attacks of "pneumonia" in 1948 and subsequently noticed that her abdomen was enlarging. She had several attacks of pain under the ribs on her left side. In January, 1951 she found several lumps on the left side of her neck. From June, 1951 she became easily tired and lacking in energy. She was first seen at the Royal Cancer Hospital on October 8, 1951. On examination she was pale, there was moderate enlargement of rubbery to firm lymph nodes in the left cervical, right axillary, and both inguinal regions. The spleen was grossly enlarged, its edge 5 cm. to the right of the midline and 5 cm. above the symphysis pubis. The liver edge was felt 10 cm. below the costal margin. A blood count showed Hb 58% (8.5 g. per 100 ml.), W.B.C. 282,400 per cu. mm., of which 97.6% were small lymphocytes. Platelets 123,000 per cu. mm. Sternal marrow showed almost complete replacement by lymphocytes.

Treatment and progress. Myleran 6 mg. daily was given from November 14, 1951. The drug was discontinued on December 18, 1951 because

scattered petechial hemorrhage were found over the front of the chest, abdomen, and shins. Hb was 64% (9.5 g. per 100 ml.), W.B.C. 281,000 per cu. mm., of which 99% were small lymphocytes, platelets 144,000 per cu. mm. On January 22, 1952 she felt better than she had for a long time. The spleen had regressed 4 cm. medially, and the liver was 6 cm. below the costal margin. All the lymph nodes had regressed and were only just palpable. Hb 59% (8.7 g. per 100 ml.), W.B.C. 21,400 per cu. mm., of which 94% were lymphocytes, platelets 89,000 per cu. mm. She remained well apart from minor infections, without further treatment until July, 1953. She was subsequently treated with radiotherapy to the spleen and radioactive phosphorus. She died on January 5, 1955.

Case 9 (Fig. 13). A spinster aged 60 years complained of progressive dyspnea and lassitude since September, 1950 and of bruising of arms and legs since December, 1950. A diagnosis of subacute myeloid leukemia was made and she was transfused on April 2, 1951 with 3 pints of blood. She was admitted to the Royal Cancer Hospital on April 4, 1951. On examination she looked frail and ill. Skin and mucosae were pale and multiple ecchymoses and petechiae were scattered over the trunk and limbs. All groups of cervical, axillary, and inguinal nodes were enlarged, some nodes up to 3 cm. in diameter. The spleen was not palpable but the liver edge was felt 9 cm. below the right costal margin. There was pitting edema up to the knees. On April 4, Hb was 44% (6.4 g. per 100 ml.), W.B.C. 270,000 per cu. mm., neutrophils 91,800, immature myeloid cells 164,780 per cu mm., platelets 15,000 per cu. mm.

Treatment and progress. Four pints of blood were transfused on April 11, 1951. From April 14 daily doses of 40, 30, 20, and 10 mg. Myleran were given and from April 18 six further daily doses of 10 mg. Her general condition improved, the liver and enlarged nodes regressed but the hemorrhagic state persisted. On April 26, Hb was 57% (8.2 g. per 100 ml.), W.B.C. 5,700 per cu. mm., 600 immature myeloid cells. One month later her general condition was good and Hb was 64% (9.5 g. per 100 ml.) but the leucocyte count was 53,000 per cu. mm., 10,600 immature myeloid cells. She died at home 2 weeks later.

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REFERENCES

Berlin, N. I., Lawrence, J. H , and Lee, H. C. 1954. *J. Lab. Clin. Med.* **44**, 860–874.

Berlin, R. 1951. *Acta Med. Scand* **139**, Suppl 252, 1–141.

Bernard, J , Mathé, G., and Najean, Y. 1955. *Sem. des Hôpitaux de Paris* **31**, No. 59.

Blackburn, E K., and King, G M 1954 *J. Fac. Radiol* **6**, 96–103.

Blackburn, E K., King, G. M., and Swan, H. T. 1956. *Brit. Med. J.* **i**, 835–837.

Black-Schaffer, B, and Stoddard, L. D 1953. *Am. J. Pathol* **29**, 413–448.

Bollog, W. 1953a. *Experientia* **9**, 268.

Bollog, W. 1953b. *Schweiz. med. Wochsche* **83**, 872–876.

Bollog, W. 1954a *Schweiz. med. Wochschr.* **84**, 393–395.

Bollog, W. 1954b. Personal communication.

Bond, W. H., Rohr, R. J , Dyke, R. W., and Fouts, P. J. 1953. *Arch. Intern Med.* **91**, 602–617.

Boyland, E., and Koller, P. C. 1954. *Brit. J Cancer* **8**, 677–684.

Burchenal, J. H. 1954. Personal communication.

Burchenal, J. H., Murphy, M. L., Ellison, R. R., Sykes, M. P., Tan, C. T. C., Leone, L A , Karnofsky, D. A , Craver, L. F., Dargeon, H. W., and Rhoads, C. P. 1953. *Blood* **8**, 965–999.

Dameshek, W. 1953a. Personal communication.

Dameshek, W. 1953b. Communication to 4th European Congress of Haematology, Amsterdam.

de Vries, S I. 1953. *Ned. Tijdsche. Geneesk.* **97**, 2330–2338.

Elson, L A. 1955. *Brit. J. Haematol.* **1**, 104–116

Elson, L. A., Galton, D. A. G., Lamerton, L F , and Till, M. 1955. Work in preparation.

Farber, S., Diamond, L K., Mercer, R D , Sylvester, R. F., Jr., and Wolff, J. A. 1948. *New Engl. J. Med* **238**, 787–820.

Fellinger, K., Mannheimer, E , Reimer, E E , and Vetter, H. 1956. *Radiol. Clinica* **25**, 1–11.

Franzen, S 1954. Personal communication.

Galton, D. A. G. 1953a. *Lancet* **i**, 208–212.

Galton, D. A. G 1953b. *Brit. J. Radiol.* **26**, 285–290

Galton, D. A G., and Till, M. 1953. Unpublished observations.

Galton, D. A. G., and Till, M. 1955. *Lancet* **i**, 425–430.

Goodman, L. S , Wintrobe, M. M , Dameshek, W , Goodman, M. J., Gilman, A., and McLennan, M. T. 1946. *J. Am. Med. Assoc.* **132**, 126–132.

Gunz, F. W. 1933 *Blood* **8**, 687–692

Haddow, A , and Timmis, G. M. 1951. *Acta Unio Intern. contra Cancrum* **7**, 469–471.

Haddow, A , and Timmis, G M. 1953. *Lancet* **i**, 207–208.

Hansen, P. 1954. "Ciba Foundation Symposium on Leukaemia Research," pp. 205–215 Churchill, London.

Hill, J. M , Spear, R. J., Marshall, G. T., and Lajous, J. R 1954. Communication to 5th International Congress of Haematology, Paris.

Hoffman, W J , and Craver, L. P. 1931. *J. Am. Med. Assoc.* **97**, 836–840.

Hutt, M. S. R., Pinniger, J. L., and Wetherley-Mein, G. 1953. *Blood* **8**, 295–314.

Kalapos, I. 1935. *Klin. Wochsche.* **14**, 861–867.

Karnofsky, D. A , Burchenal, J. H , Armistead, G. C., Southam, C. M., Bernstein, J. L., Craver, L. F., and Rhoads, C. P. 1951. *Arch. Intern. Med.* **87**, 477–516.

Klima, R , von Beyreder, J., and Herzog, E. 1954 *Wien klin. Wochschr.* **35**–6, 3682–3685.

Koranyi, A. 1912. *Berlin klin. Wochschr.* **49**, 1357–1358.

Kurrle, G. R. 1955. *Med. J. Australia* **42**, 636–641.

Lange, R. D , Moloney, W. C., and Yamawaki, T. 1954. *Blood* **9**, 574–585.

Lawrence, J H. 1954. *Med. Clin. N. Amer.* **38**, 525–540.

Lawrence, J. H., Low-Beer, B. V. A., and Carpender, J. W. J. 1919. *J. Am. Med. Assoc.* **140**, 585–588.

Lawrence, J H , Dobson, R. L., Low-Beer, B. V. A., and Brown, B. R. 1948. *J. Am. Med. Assoc.* **136**, 672–677.

Ledlie, E. M. 1953. *Brit. J. Radiol.* **26**, 290–295.

Levin, W. C., Thaddeus, A. P., Vaughan, J. R., Jr., and Tsai, S. Y. 1954. *J. Lab. Clin. Med.* **44**, 890.

Lissauer, 1865. *Berlin klin. Wochschr.* **2**, 403–404.

Louis, J., Best, W. R., and Limarzi, L. R. 1954 *J. Lab. Clin. Med.* **44**, 893–894.

McGavran, C W. 1938 *Ann. Intern. Med.* **12**, 396–402.

Maingot, G , Girard, L , and Bousser, J. 1938. *Le Sang.* **12**, 569–582.

Medical Research Council & Nuffield Foundation Joint Report. 1954. *Brit. Med J.* **1**, 1223–1227.

Meyer, L M , Schwartz, S. O., Sawitzky, A , Beyers, M. R., Ritz, N. D., Brahin, C., Diefenbach, W., Kleinschmidt, W., and Friedman, I. 1952. *Acta Med. Scand.* **144**, Suppl. 272, 3–36.

Minot, G. R., and Isaacs, R. 1924 *Boston Med. Surg. J.* **191**, 1–9.

Minot, G R., Buckman, T. E., and Isaacs, R. 1924 *J. Am Med. Assoc.* **82**, 1489–1494.

Moeschlin, S., Meyer, H , and Lichtman, A. 1954. "Ciba Foundation Symposium on Leukemia Research," pp 216–230.

Moffit, H. C., and Lawrence, J. H. 1919 *Ann. Internat Med.* **30**, 778–790

Moloney, W. C., and Lange, R. D. 1954 *Blood* **9**, 663–685.

Moloney, W. C., and Takashi, F. 1954 *Acta Haematol. Japan* **17**, 120–121.

Nabarro, J. D. N. 1955. Personal communication.

Osgood, E. E. 1951. *Arch. Intern. Med.* **87**, 329–348.

Osgood, E. E., and Seaman, A. J 1952 *J. Am. Med Assoc* **150**, 1372–1379.

Paterson, E., and Kunkler, P. 1954. "Ciba Foundation Symposium on Leukaemia Research," pp. 231–243. Churchill, London.

Paterson, E., Haddow, A , Thomas, I A , and Watkinson, J. M. 1946. *Lancet* **1**, 677–682.

Paterson, E., Haddow, A., Thomas, I. A , and Watkinson, J. M. 1947. "Approaches to Tumor Chemotherapy," pp. 401–415. American Association for the Advancement of Science. Washington, D C.

Pearson, O H., Eliel, L. P., Rawson, R. W., Dobriner, K , and Rhoads, C. P 1949. *Cancer* **2**, 943–945.

Petrakis, N. L., Bierman, H. R., Kelly, K. H., White, L. P., and Shimkin, M. B. 1954. *Cancer* **7**, 383–390.

Rider, W D. 1954. Personal communication.

Senn, N. 1903. *N.Y. Med. J.* **77**, 665–668

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Shimkin, M. B, Mettier, S. R, and Bierman, H. R. 1951. Ann Internal Med. 35, 194–212.

Shimkin, M. B, Lucia, E. L, Opperman, K. C, and Mettier, S. R. 1953. Ann. Internal Med. 39, 1254–1266.

Silva, M. S., Burchenal, J. H., and La Due, J S. 1954. Rev. Brasil. cirurg. 27, 395–452.

Silverberg, J H., and Dameshek, W. 1952. J. Am. Med. Assoc. 148, 1015–1021.

Timmis, G. M. 1948 British Patent Application 20, 464.

Tivey, H. 1954. Am. J. Roentgenol. Radium Therapy Nuclear Med. 72, 68–93.

Turesson, D. 1953 Svenska Läkartidn 50, 1025.

Videbaek, A. 1955 Acta. Med Scand. 151, 295–306.

Wilkinson, J. F. 1953. Proc. Roy Soc. Med. 46, 685–692.

Wintrobe, M. M., and Hasenbush, L. L 1939. Arch. Intern. Med. 64, 701–718.

Wintrobe, M M, Cartwright, G. E, Fessas, P, Haut, A., and Altman, S. J. 1954. Ann. Internal Med. 41, 447–464.

Zarafonetis, C J. D., Shay, H, and Sun, D. C H. 1955. Cancer 8, 512–522.

THE USE OF 6-MERCAPTOPURINE IN THE TREATMENT OF LEUKAEMIA

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THE effects of 6-mercaptopurine therapy in human leuk-
aemia are well known (Burchenal *et al.*, 1953; Conference on
6-mercaptopurine, New York, 1954; Hayhoe, 1955; Marinone
and Introzzi, 1955). The following remarks are based on
observations made in collaboration with Dr. M. Till and Dr.
E. Wiltshaw on 18 patients suffering from acute leukaemia
and on 12 patients in the terminal stages of chronic myeloid
leukaemia. The detailed results, which agree well with those
in the literature, will be published elsewhere and the present
intention is to outline the circumstances in which 6-mercapto-
purine can induce remission in leukaemia and to define the
limitations of the drug. Neither series is representative
because there were only 3 children among the 18 "acute"
cases, and because 11 of the 12 "chronic" patients had
relapsed while receiving continuous treatment with Myleran
which had effectively controlled their disease for periods up
to 3 years. Further, only 1 of the cases of acute leukaemia
was lymphoblastic, the cell type being myeloblastic in 14, un-
differentiated in 2, and in 1 case the type has not been estab-
lished. Only 3 of the "acute" patients had received previous
treatment, in 2 cases with cortisone, in one with Myleran.

Fig. 1 indicates the approximate range of activity of 6-
mercaptopurine in leukaemia, compared with that of the
folic-acid antagonists and of the cytotoxic drugs. 6-Mercapto-
purine (and 6-chloropurine) are the only drugs of practical
value which are effective both in acute and chronic leukaemias.
They are, however, rarely effective in chronic lymphatic
leukaemia. They are effective in acute leukaemia in children

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TYPE OF LEUKAEMIA		CYTO-TOXIC DRUGS	FOLIC-ACID ANTAGONISTS	6-MERCAPTO-PURINE
Acute		—	+	+
Chronic	Myeloid	+	—	+
	Lymphocytic	+	—	—

FIG. 1.   Response to three groups of antileukaemic agents.

and adults, whereas folic-acid antagonists are rarely of value in adults. There is no cross-resistance between the two groups of drugs. In early chronic myeloid leukaemia, 6-mercapto-purine will induce remission but has not been used long enough to know whether it has advantages over other forms of therapy (Burchenal, 1956).

Acute Leukaemia

6-Mercaptopurine differs from corticotrophin, the steroid hormones and folic-acid antagonists in its slow action. Be-cause of this it cannot be used as the only means of therapy in fulminating cases in which the risks to life are great if remission is not induced rapidly. Fig. 2 indicates the hazards that threaten life during the induction of remission in acute leukaemia.

1. Hazards during the first week of therapy

When the peripheral blast count rises quickly the patient may succumb to acute intoxication. One of the patients in the present series died from this cause, the peripheral blast count having increased from 50,000 to 590,000 per mm.^{3} in the 7 days before death.

With increasing replacement of normal bone marrow by leukaemic tissue the neutrophil leucocyte and platelet counts fall. The latter results in haemorrhagic events while the

former exposes the patient to infection to which little or no resistance is offered. Two patients succumbed to uncontrollable haemorrhage in the first week of treatment.

2. Hazards resulting from the drug

The drug itself may depress the neutrophil and platelet counts with the same consequences that follow spontaneous
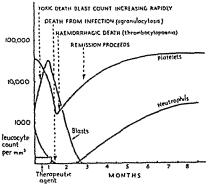
FIG. 2. Hazards during induction of remission in acute leukaemia.

neutropenia or thrombocytopenia. Two patients succumbed to overwhelming infection 5 weeks after starting treatment with 6-mercaptopurine.

3. Early hazards survived. Remission not induced.

Although remission may begin as early as 3 weeks after starting treatment, in some cases the onset of remission may be delayed as long as 8 weeks after the first dose of 6-mercapto-purine, while in others haematological changes characteristic of early remission are observed but prove to be abortive. One

patient died after continuous treatment for 9 weeks with
6-mercaptopurine without securing a remission. Another
patient was treated for $11\frac{1}{2}$ weeks with 6-mercaptopurine
alone, and in combination first with Myleran and then with
cortisone. Seven months later he was again treated with
6-mercaptopurine and cortisone. This patient lived and
worked for 18 months, but required blood transfusions every
4 to 8 weeks; these rather than the drugs seemed responsible
for keeping him alive.

4. Remission induced

True remissions are recognized by continued rise in neutro-
phil and platelet counts and by reversal of the falling haemo-
globin trend. Blood transfusion can be dispensed with and
the danger from haemorrhage and infection passes. Leuk-
aemic infiltrations and their clinical manifestations disappear;
the bone marrow is cleared of blasts and active regeneration of
myeloid and erythroid tissue and of megakaryocytes takes
place.

The likelihood of inducing remission with 6-mercaptopurine
appears to depend more on the peripheral leucocyte count
than on the age of the patient or on the cell-type. Only 1 of
the 6 patients with high leucocyte counts responded to treat-
ment and the remission lasted only 8 weeks. In contrast 9 of
the 12 patients with subnormal peripheral leucocyte counts
secured remissions.

Because of the slow action of 6-mercaptopurine, only 7 of
our 18 patients were thought suitable for treatment with this
substance alone. In 4 cases remission ensued between 3 and 8
weeks after treatment was begun (Fig. 3). The remaining 3
patients who received 6-mercaptopurine alone died without
achieving remission.

In 9 instances, the patients were considered unlikely to
benefit from 6-mercaptopurine therapy alone, because of the
severity of their illness. They were treated with a combina-
tion of 6-mercaptopurine and cortisone, prednisolone or
corticotrophin. Three died before remission could be induced

and in one remission was incomplete and shortlived. Remissions were obtained in the remaining 5 patients; 2 were adults and 3 were children. The hormone appeared to play
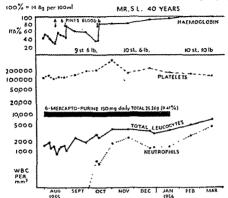
FIG. 3. Acute leukaemia. Effect on blood counts of treatment with 6-mercaptopurine alone *Note delay before remission was induced. Treatment was discontinued because marrow was hypoplastic.*

little part in the induction of remission in one case (Fig. 4) but in 2 of the children combination therapy seemed to maintain remission better than either drug used alone.

One patient received a combination of 6-mercaptopurine and Myleran; half the standard dose of each drug was used. Remission followed, but the possibility that it was unrelated to therapy cannot be excluded owing to certain atypical features in the curves of haemoglobin level and neutrophil counts. A second patient, already mentioned, received a combination of Myleran and 6-mercaptopurine without benefit.
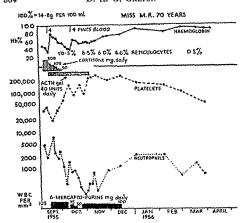
FIG. 4. Acute myeloblastic leukaemia. Effect on blood counts of combined treatment with corticotrophin, cortisone and 6-mercaptopurine. Note delay before remission was induced.

Duration of remissions

Two of the 3 children were maintained in good health for 10 months and 1 year. The third, who had relapsed after successful treatment for 6 months with cortisone, responded for only 8 weeks to a combination of 6-mercaptopurine and corticotrophin.

In the 7 adults, remissions lasted from 4 to 10 months and 4 patients are in remission at the time of writing. Only one patient secured a second remission, which lasted 5 months; there was no response to a third course of 6-mercaptopurine.

To the clinician, the excellent health enjoyed by patients in remission gives satisfaction but this is offset by the knowledge

that relapse is inevitable with the limited therapeutic resources available.

Chronic Myeloid Leukaemia in Terminal Relapse

Resistance to Myleran was shown by progression of clinical and haematological manifestations of disease which could not be controlled by increasing the dose of Myleran. Before 6-mercaptopurine became available such patients rarely survived more than 6 weeks.

In 2 cases improvement in well-being, reduction in spleen size, fall in myeloblast count and spontaneous rise in haemoglobin level followed treatment with 6-mercaptopurine. In one case the fall in platelet count was arrested; in the other it was reversed. Both patients continued to work for another 5 months before they succumbed. In two cases, 6-mercaptopurine therapy reduced the size of the spleen, abolished the intoxication associated with myeloblastosis; but spontaneous rise in haemoglobin did not occur. Both patients required supportive therapy with blood transfusions, steroid hormones and in one case splenectomy. They lived 6 and 8 months after mercaptopurine therapy was begun. A fifth patient, who had become resistant to Myleran, was treated for 1 week with 6-mercaptopurine after which she responded again to Myleran for 7 months, only to succumb to intercurrent infection. Seven of the 12 patients derived no benefit from treatment with 6-mercaptopurine. As in acute leukaemia, the significance of these results, in themselves of so little importance, is the demonstration of the potential reversibility of a catastrophe hitherto regarded as inevitably fatal.

REFERENCES

BURCHENAL, J. H. (1956). *Current Res. in Cancer Chemotherapy*, 4, 13.

BURCHENAL, J. H., MURPHY, M. L., ELLISON, R. R., SYKES, M. P., TAN, T. C., LLOYD, L. A., KARNOFSKY, D. A., CRAVER, L. F., DARGEON, H. W., and RHOADS, C. P. (1953). *Blood*, 8, 965.

Conference on 6-Mercaptopurine (1954). *Ann. N.Y. Acad. Sci.*, 60, 185–507.

HAYHOE, F. G. J. (1955). *Lancet*, 2, 903.

MARINONE, G., and INTROZZI, P. (1955). La Terapia delle Leucemie, *Bibl. Haemat.*, 21, suppl.

Busulphan (Myleran) in Chronic Myelogenous Leukemia

E. WILTSHAW AND D. A. G. GALTON*

BUSULPHAN was first used in the treatment of chronic myelogenous leukemia six years ago and it is opportune to try to compare its effects on survival with that of conventional radiotherapy. We have collected survival times of 81 patients treated with radiotherapy alone or with busulphan alone or with both methods used consecutively.

CLINICAL MATERIAL

The records of 115 patients who attended the Royal Marsden Hospital with a diagnosis of chronic myelogenous leukemia between January 1936 and July 1956 were studied. Cases were discarded when the diagnosis was in doubt, when details of therapy were incomplete or when follow up was inadequate. The group of patients with leukemia attending this hospital may not reflect the distribution of the disease in the general population. Most patients were referred from other hospitals for special care, some had already failed to respond to treatment and others were sent in response to efforts made to collect patients for trial of a new therapeutic agent. Nevertheless the age distribution of our patients (fig. 1) accords well with that found by others. Of the 81 patients whose records were adequate 46 were females and 35 were males. Two males and seven females are alive and well (August 1956).

ESTIMATION OF SURVIVAL

In published series, survival is recorded from the time of appearance of first symptom, from the date of diagnosis or from the date of first treatment to death. In chronic myelogenous leukemia onset is usually insidious and often cannot be fixed within six months. To record onset from first treatment is unsatisfactory because indications for starting therapy are so variable. The date of diagnosis bears no constant relation to the onset of disease. Thus in our series a few patients were diagnosed following routine blood counts when volunteering as blood donors, others were discovered while under treatment for an apparently unrelated illness; in some cases episodes in the history suggestive of splenic infarction or priapism indicated that the disease was present at an earlier date; occasionally a definitive diagnosis could be made only in retrospect. One patient receiving treatment for tuberculosis developed leukemia over a period of two years. The transition was so gradual that no date of onset could be defined and the case has been excluded from the survey.

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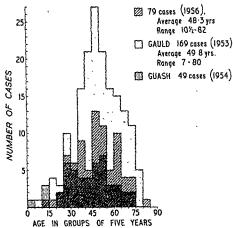
Fig 1 Age distribution of 79 cases of chronic myelogenous leukemia compared with that found by Gauld et al (1953) and Guash (1954).

In analyzing survival times, onset has been recorded both from the date of appearance of first symptom and from date of diagnosis recognizing that each method is at best an approximation.

TYPES OF THERAPY

Over the twenty-one year period from which the 81 cases have been drawn there has been little continuity of care; the practice of general medicine has changed radically and several clinicians have treated the patients The conditions under which patients receiving busulphan were treated differed greatly from those obtaining during the early years of the survey period and their significance cannot be readily assessed. However, median survival of patients diagnosed before and after 1950 is the same.

When busulphan was first used large doses were given and shortened life span in several cases. Some patients were treated with arsenic, urethane, nitrogen mustard, and para-dimethylaminostilbene. 6-Mercaptopurine was given to seven patients within the last eight months of life which may have prolonged life by some four months in three patients. Because of the small number of cases, doubtful efficacy and difficulty of assessment these drugs have been ignored in this survey. Radiotherapy consisted of irradiation to the spleen in most cases

and to long bones or to the whole body in a few; two patients were treated with radioactive phosphorus.

RESULTS

Survival time from first symptom and from diagnosis have been plotted for the whole group and for ten subgroups according to sex and treatment received.
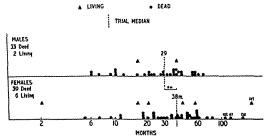
FIG. 2. Chronic myelogenous leukemia in 80 patients Survival from first symptom related to sex. (All types of therapy). Note Trial median for all cases, 32 months.
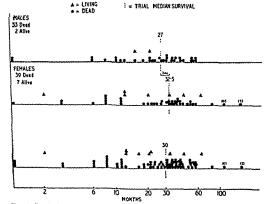
FIG. 3. Survival from month of diagnosis in 81 patients suffering from chronic myelogenous leukemia.

The subgroup 'no therapy' includes patients who received treatment other than busulphan or radiotherapy. The subgroup "x-ray therapy and myleran" refers to patients given both methods of treatment at some time during the course of their disease. Radiotherapy and busulphan have not been used simultaneously in any patient. For each group a trial median survival has been indicated for the dead patients.

Survival related to sex (figs. 2 and 3). Fifty per cent of thirty-nine dead females lived more than 38 months from the onset of their first symptom, and 32.5 months from the date of diagnosis For 33 dead males the corresponding figures are 29 and 27 months. There was an excess of females in the older age groups but analysis shows that survival time was not related to age at diagnosis in either sex.

Survival related to therapy (figs. 4 and 5). Of the 6 females in the "no therapy" group 3 lived longer than 18 5 months from the date of diagnosis; of the 4 males 2 lived longer than three months. Four of the seven dead females who received busulphan only and 3 of the 5 dead males lived 31 months or more.

Of the 18 females given x-ray therapy alone 9 lived more than 29 months and 9 of 17 males lived 26.5 months or more. Four of the 8 females who received busulphan and radiotherapy lived more than 46 months and 4 of the 7 males lived 34 months or more.
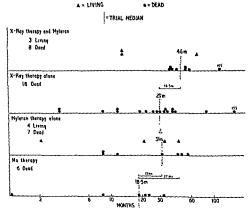
FIG. 4. Chronic myelogenous leukemia in 46 female patients. Survival from diagnosis related to the type of therapy
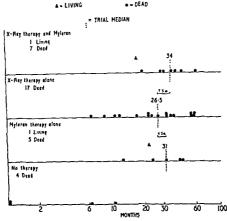
FIG 5 Chronic myelogenous leukemia in 35 male patients Survival from diagnosis related to the type of therapy.

DISCUSSION

From figures 2 and 3 it is evident that females suffering from chronic myelogenous leukemia tend to live longer than males.

Figures 4 and 5 show that type of therapy has little influence on survival but that patients treated with both radiotherapy and busulphan lived rather longer than those treated by either method alone and that this difference is greater for females than for males. Crosby et al. found that the average dose per kilogram of radioactive phosphorus necessary to keep the disease under control was significantly less in females than in males.

Hitherto we have been unable to advocate busulphan as a first line of treatment because it was possible that long courses of the drug might cause irreversible bone marrow damage. However, this study shows that the life span of patients treated with busulphan alone is similar to that of patients treated by radiotherapy alone. When both radiotherapy and busulphan are used survival may be slightly increased.

ACKNOWLEDGEMENTS

HYPOPHYSECTOMY FOR CANCER

By Mr. W. P. GREENING (Royal Marsden Hospital)

The purpose of this paper is to discuss the effect of beta radiation, using yttrium90, on the human hypophysis; and to show whether the transphenoidal insertion of yttrium90 rods, in an attempt to destroy the pituitary gland, is either a justifiable or worthwhile procedure.

We think that interstitial irradiation of the pituitary has a definite place in the treatment of selected cases. It is not a substitute for ablative surgery but an alternative procedure for those patients where a high operative mortality is expected, if they are subjected to a major operation.

Complete destruction of the gland is desirable but not essential, and worthwhile remissions may be obtained, with relief of symptoms, when a large part is destroyed.

Since metastatic cancer of the breast inevitably terminates in death, it would be as well to review the different methods of treatment at present available.

Galton, in 1950, published a report dealing with 70 cases of breast cancer treated by androgens. All his patients were in an advanced stage of the disease and were completely unselected.

He reported a failure rate of 50%, a success rate, described as successful or moderately successful, giving a combined total of over 40%. He said that of the 30 patients deriving benefit from the treatment, 21 obtained a remission lasting six months or more, 10 over one year and 3 over eighteen months. His maximum success rate was between the ages of 45 and 50, but a similar, though smaller, peak occurred between the ages of 35 and 40

Improvement following œstrogen therapy in the post-menopausal patients is of a similar order.

Cade, in 1954, in an assessment of the results of adrenalectomy in a series of 36 patients, gave a complete absence of response in 29%, a very successful rate of 23% and a less successful, though still improved, rate of 31%. He gives an extended survival of up to twenty months, and remarks that successful results were achieved in about 52% of the patients.

The results of hypophysectomy as reported by Luft (Olivecrona and Luft, 1957), Pearson *et al.* (1956) and Kennedy (1956) show a slight increase in the success rate and a rate corresponding favourably with that of adrenalectomy. However, in Luft's series hypophysectomy was achieved in only a few cases, and there has been a considerable improvement in his recent results, due to complete removal of the gland. The series reported by Kennedy was carefully selected, and this may account for the high rate of remission.

This remarkable uniformity of results obtained by varying methods of treatment, indicates that hormone responsiveness in breast cancer is limited to half of the patients.

Only 5 women out of 10 will benefit from *any* method which alters the endocrine balance.

These facts make it necessary to balance the magnitude of the treatment against the result that may reasonably be expected.

A mortality of 6% in both adrenalectomy and hypophysectomy, and a morbidity rate in the latter operation of similar proportions is inevitable. In both operations adrenal ⸺⸺ ⸺⸺ ⸺⸺ ⸺⸺ ⸺⸺ in a significant number of cases. Any ⸺ ⸺ ⸺ ⸺ ⸺ ⸺ ⸺ " 'he patient and accompanied by a low

It seemed that a small group of patients with metastatic breast cancer who, because of their poor general condition were considered unsuitable for ablative surgery, might benefit from interstitial irradiation of the pituitary, and we were, in the first place, trying to devise a method of either completely or almost completely destroying the gland, without the hazards or inconvenience of a major operation.

50 patients, unsuitable or unfit for adrenalectomy, and possibly therefore for surgical hypophysectomy, were treated by interstitial irradiation of the pituitary using unscreened radio-active gold. As this form of treatment was regarded only as temporary palliation, every effort was made to avoid complications caused by damage to the neighbouring structures, and to select carefully the patients who might be hormone responsive.

The activity of the gold seeds used in these cases was increased from a low value at first up to 20 mc. towards the end of the series. The destruction of the gland was inadequate but the complications were few. They were damage to the parasellar nerves in 3 cases, two VI nerve and one III nerve palsy. There was one case of cerebrospinal rhinorrhœa.

The success rate and duration of the remission so produced was not considered good enough to justify continuing on these lines. For the last 30 cases we have used yttrium90, because it is a pure beta emitter and has a short half-life of 64 hr. The yttrium90 rods used are made of sintered yttrium oxide, and are 1·3 mm. diameter, and 4·0 mm. long. Each source has an activity of approximately 3 mc. at the time of implantation. Investigations by a combination of film and ionization chamber techniques show that along the perpendicular axis, the half-value layer of tissue is 0·7 mm. It has also been shown that with an activity of 3 mc. the total dose is approximately 70,000 rad at 3 mm. from the rod; the necrotic boundary has been found to lie at this distance in a number of post-mortem sections of the hypophysis, and is a spherical volume of complete necrosis surrounded by normal tissue. The boundary transition between normal and necrotic tissue is extremely narrow.

In this series a success rate of 40% has been obtained, a figure not dissimilar to those produced by endocrine therapy and ablative surgery, although it is already apparent that the length of the remission is probably shorter.

The immediate operative mortality has been 3%, but few of these cases would have been even remotely considered fit for adrenalectomy.

One patient developed cerebrospinal rhinorrhœa and one died of meningitis eight weeks after operation. There was no apparent cause for this at post-mortem, the floor of the sella was intact and the pituitary almost completely destroyed. There are two unilateral III nerve palsies in this series, and one unilateral optic atrophy. The urinary gonadotrophin in the cases treated by yttrium90 is estimated before, and at monthly intervals after, the operation, using the method developed in the Clinical Endocrinology Research Unit in the University of Edinburgh, and we are indebted to Dr. L. A. Loraine and Professor Boyland for help and advice with these estimations.

The biological assay is based on the method of Klinefelter, Albright and Griswold, 1943, depending on the enlargement of the uterus of intact immature mice, 20–22 days old weighing 8–10 grams.

We find that 80% of cases show that gonadotrophin is completely eliminated from the urine post-operatively, in amounts detectable by this bio-assay.

In considering the technique of implanting the yttrium, the following criteria must be satisfied:

(1) There must be accurate insertion into the hypophysis.

(2) The dose must be sufficient to cause necrosis of the gland but not to affect the surrounding structures.

(3) It should be capable of being done quickly and easily without damage to the floor of the sella turcica.

(4) Unless these conditions are completely satisfied the operation should not be undertaken.

We use an image intensifier working in two planes. In the lateral view there is no difficulty but we find that a modified Townes' view in the antero-posterior projection is of great value in locating the sella.

Following the operation, in most cases little discomfort is experienced, but in some, aspirin or codeine is necessary to relieve headaches for the first few post-operative days.

The patient is kept on antibiotic cover both before and after the procedure.

Cortisone is withheld until there are signs of adrenal insufficiency. They appear at about the seventh day and are loss of appetite, nausea and vomiting. They cease as soon as cortisone is administered.

Transient diabetes insipidus occurs in most cases between the second and third weeks.

We think that damage to the chiasma or surrounding structures can be completely eliminated by accurate placing of the rods under fluoroscopic control.

REFERENCES

CADE, S (1954) *Ann. R. Coll. Surg. Engl.*, **15**, 71.
GALTON, D A G. (1950) *Brit. J. Cancer*, **4**, 20.
KENNEDY, B. J. (1956) *Amer. J. Med.*, **21**, 721.
KLINEFELTER, H. F., Jr., ALBRIGHT, F. and GRISWOLD, G. C. (1943) *J. clin. Endocrin.*, **3**, 529.
OLIVECRONA, H., and LUFT, R. (1957) *Ann. R. Coll. Surg. Engl*, **20**, 267
PEARSON, O H., RAY, B S, HARROLD, C. C., WEST, C. D., LI, M. C., MACLEAN, J. P., and LIPSETT, M. B. (1956) *J. Amer. med. Ass.*, **161**, 17.

Effect of Radioactive Colloidal Gold on the Mast Cells of Bone Marrow
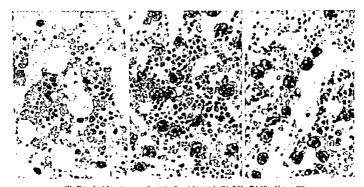
AN increase in the number of mast cells has been reported in the thymus, spleen and bone marrow of mice exposed to long-continued gamma irradiation[1]. Hypoplasia of the bone marrow has been reported in animals and man following the administration of radioactive colloidal gold (gold-198)[1], but no reference is made to any change in the mast cells. In the course of an investigation of the general histopathological effects of colloidal gold-198 administered to rats, we have found an increase in the mast cell population of the bone marrow.

The colloidal gold was supplied by the Radiochemical Centre, Amersham, and consisted of a colloidal suspension of metallic gold stabilized with gelatin. The particle-size was stated to be approximately 20–25 mμ. Twenty-one male rats, of the

tion of fat cells. In addition, the number of mast cells was found to be increased and this increase was still apparent in the bone marrow of the animals which died at all times up to 120 days. In the bone marrow sections of each animal studied, the number of mast . . . . nated . . . . . . each . . . . . n the . . . . 'erage number of mast cells in ten high-power fields was 257 (range 159–329) in the irradiated animals compared with 32 (range 18–39) in normal animals.

In the irradiated animals the mast cells were more abundant in the marrow in the middle of the femur shaft and were located predominantly along the walls of the sinuses. They were consistently larger, and vacuolation and dilaceration were more conspicuous than in the mast cells of normal animals (Figs. 1–3). No evidence of mitosis was found among mast cells

(1) Normal rat bone-marrow; six mast cells can be seen in this field. Toluidine blue. × 350
(2) Bone marrow from an animal which died 90 days after injection of gold-198. The mast cells are swollen and dilacerated. Toluidine blue. × 350
(3) Bone marrow from same animal as (2). Mast cells are located along the sinus wall. Toluidine blue. × 350

highly inbred 'August' strain, weighing 80–90 gm., were given 15 μc. per gm. of body-weight of colloidal gold-198, seventeen animals being injected intravenously and four intraperitoneally. Three animals died within 30 days of injection, fourteen between 60 and 90 days and four between 90 and 120 days after injection. Autopsies were performed on nineteen of the animals, and in every case extensive necrosis of the liver was found. In twelve animals hæmorrhage into the gastro-intestinal tract, lungs and testes was observed, and in seven animals a considerable amount of fluid was present in the pleural or peritoneal cavities. Sections were prepared from liver, spleen, lung, kidney, testis and bone marrow (femur).

The bone marrow of the animals which died within thirty days of injection showed the typical recovery pattern which is observed after irradiation; the total cell population was below normal, the sinuses were dilated and congested and there was a high propor-

in any of the many sections examined, and there were no significant differences between the animals injected intraperitoneally and intravenously. The mast cell population of the spleen was apparently unchanged, although histological signs of irradiation damage were present; the thymus was not examined.

We can offer no plausible explanation for this increase in mast cells. It seems, on morphological grounds alone, that the mast cells are produced locally, and their increase does not result from an invasion of cells from outside. The remarkable absence of mitosis suggests that the increase is not the consequence of a proliferation of existing mast cells but more probably results from transformation and differentiation of primitive reticular cells; the localization of the cells along the sinus walls is highly suggestive of this.

Mast cells are considered to be related to the production of heparin[3], and the occurrence of a

section describes the various features manifested by the genetic material of the malignant cell; and their possible role in the genesis of cancer is discussed.

Mitosis and chromosome behaviour in tumour cells

The number of dividing cells is usually high in tumours, and the high mitotic index is a reliable measure of the growth of a particular tumour. The pioneer investigators of tumour pathology were already aware of the significance of mitosis and noted the irregularities which occurred during this process. Hansemann, (1890, 1891, 1892, and 1906) described the various abnormalities and concluded that the fundamental change in a tumour cell consists of a disturbance in cellular balance, either between chromosomes or between cytoplasm and nucleus. He assumed that the abnormal mitosis is the mechanism by which this is brought about. He considered that the cellular balance must be maintained for proper differentiation to occur (*prosoplasia*) while imbalance results in cancerous behaviour (*anaplasia*) (see·Whitman, 1919).
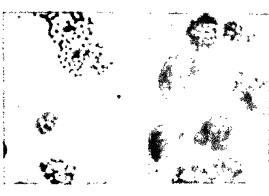
Many cytological studies have been carried out since the work of Hansemann, and much information is now available about the abnormal chromosome behaviour in tumour cells (*see* Ludford, 1930b; Koller, 1947a). The abnormalities of tumour mitosis can be divided into two classes: (1) Those which affect the chromosomes, and (2) those which interfere with spindle development.
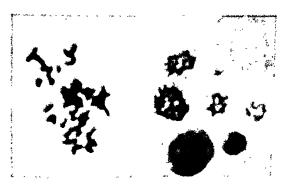
The most frequent irregularity is the stickiness of chromosomes, which can interfere with proper chromatid separation during anaphase or telophase of mitosis (Figs. 45, 46 and 49a and b). The configuration of "sticky" chromosomes is

Fig. 45. C... ... ... in a cell of an early anaphase. The daughter ...

similar to that of the closely paired homologous chromosomes during meiosis (gamete formation) This similarity led Farmer, Moore and Walker (1904) to conclude that carcinogenesis is a process similar to gametogenesis, occurring abnormally in somatic tissue.

Mitotic spindle disturbances are also frequent in dividing tumour cells (Figs. 47*a* and *b*, 48, and 49*c*). The spindle may be completely suppressed as a result of which the daughter chromosomes fail to move towards the opposite poles and a polyploid cell is formed (Ludford, 1930a). When a multipolar spindle is present the chromosomes are able to move from the equatorial plate but they will be
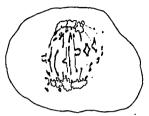
FIG 46 —Sticky chromosomes at telophase simulating the bivalent configuration of meiosis (cervical carcinoma)   (Magnification × 2,000)
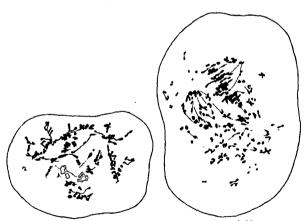
FIG. 47 —Polyploid tumour cells with multipolar mitotic spindle and sticky chromosomes (cervical carcinoma).   (Magnification × 2,000.)
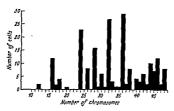
FIG. 54.—Histogram showing the distribution of cells with different chromosome numbers in a human carcinoma of the colon. Note that the variation occurred in the subdiploid direction (Koller, 1947a).

only 37 per cent of the 92 cells analysed had the diploid number. The variation in the number of chromosomes was similar to that found in embryonic tissues (Hsu, 1952). The author studied cell populations of more than 100 human effusions, and found a considerable variation not only in effusions of different patients, but in those of the same patient taken at different intervals (Fig. 53a and b, 56, and 57a and b). The data indicate that the range of variation in chromosome number depends to a great extent on the environmental conditions in the fluid (Koller, 1956).
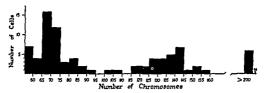
FIG. 55.—Distribution of chromosome numbers of a human liposarcoma cultured *in vitro* by Hsu (1954b). Note that the variation occurred in the hyperdiploid direction.

Mammals

Variation in chromosome numbers was also observed in somatic cells of mammals, for example in the omentum of the cat, in the foetal membrane of the pig, and in embryonic rat tissues (see Beatty, 1954). The author also encountered cells with abnormal chromosome number in the bone marrow of adult rats. By employing an improved tissue culture technique, Hsu and Pomerat (1953) were able to analyse chromosomes in heart, liver, lung and skin of embryos of the mouse, guinea pig and the dog. The heteroploid numbers ranged from 39 to 46 in the mouse, the
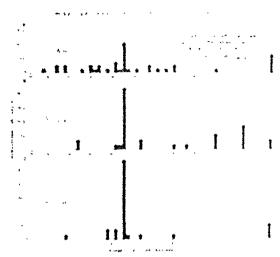
Fɪɢ. 48.—Telophase in a polyploid cell The four chromosomally unbalanced daughter nuclei remain in association by sticky chromosome bridges (cervical carcinoma) (Magnification × 2,000.)

distributed irregularly among the several daughter nuclei. These abnormalities are common in both spontaneous and induced tumours.

The mitotic irregularities produce cells with "cellular imbalance" postulated by Hansemann to be the cause of cancer. This idea was carried further by Boveri (1929) who stated that the abnormal distribution of chromosomes is the primary cause of cancerous behaviour. The classical experiments with double-fertilized sea-urchin eggs convinced him that the chromosomes of a cell have different values. He observed that an abnormal combination of the chromosomes, due to a multipolar spindle, is followed by an increased rate of uncoordinated mitoses in the developing larvae of the sea-urchin. Because the irregular groups of cells resembled malignant tumour tissue, Boveri suggested that cancerous growth might originate in a somatic cell which had acquired a defective chromosome content by multipolar division. The analysis of chromosome number in tar-induced skin tumours of mice led Winge (1930) to the same conclusion.

The incidence of mitotic abnormalities

A quantitative analysis of various human carcinomas has been undertaken to determine if the abnormalities are of general occurrence. In Tables I and II it is shown that the incidence differs significantly in different tumours and in different regions of the same tumour. Comparison of data obtained from biopsies taken from the same region of a tumour at different intervals suggests that the frequency of abnormally dividing cells in the same cell population can also fluctuate. A very high incidence of abnormal mitosis was found in necrotic regions, while mitotic disturbances were rare, or completely absent, in the actively growing periphery of tumour cords. On account of this variability, which cannot be predicted, it is not
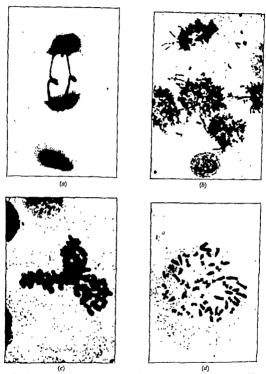
FIG. 49 —(a) Late anaphase in a tumour cell showing two chromosome bridges due to stickiness in the terminal segment The tumour has been induced in a rat by an aromatic mustard (see Table III, CB1044, tumour A), (b) synchronous division in tumour cells of a human effusion, showing stickiness of chromosomes, and the presence of multipolar spindle, (c) metaphase of mitosis in a squamous cell of carcinoma cervix showing a three-armed equatorial plate, indicating a tri-polar spindle; (d) metaphase in a dividing cell of Walker–carcinoma 256 in the rat The chromosomes in normal cells are rod-shaped, in the tumour cells v-shaped and ɪ-shaped chromosomes are present. (Magnification × 2,200 except 49c: × 2,600.)
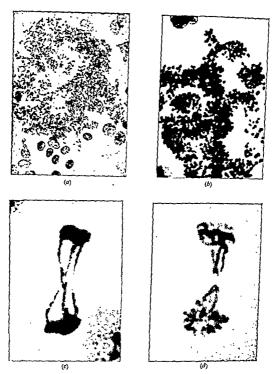
FIG. 57.—(a) Dividing giant cell in a specimen of human ascites, in which more than 800 chromosomes are present; (b) synchronous mitoses in a giant multinucleate cell, showing the mixing up of equatorial plates and chromosomes; (c and d) chromosome bridges in a rat tumour induced by an aromatic mustard (see CB1048, tumour A in Table III). The bridges break at late anaphase (c) and the broken ends become joined at telophase (d), which initiates another cycle of bridge formation. (Magnification: (a) × 400, (b) × 2,200, (c) and (d) × 2,600)

permissible to use the frequency of cells with abnormal mitosis as a criterion for estimating either the growth-rate or the degree of malignancy of a tumour. Recently the suggestion has been put forward that the presence of tri-polar mitosis in suspected carcinoma *in situ* in the squamous-cell epithelium of the cervix indicates malignancy (Therman and Timonen, 1954).

TABLE I

THE FREQUENCY OF MITOTIC ABNORMALITIES IN CELL POPULATIONS TAKEN FROM THE PERIPHERY AND CENTRE OF THE SAME TUMOUR CORD

(Koller, 1947a)

Tumour	Region (multiple biopsies)	Periphery		Region (multiple biopsies)	Centre	
		Number of dividing cells	Percentage of abnormal dividing cells		Number of dividing cells	Percentage of abnormal dividing cells
Squamous-cell carcinoma cervix No. 41 . . A	151	3·97	E	51	15 67	
B	97	5·15	F	48	20·83	
C	241	5·80	G	93	24·74	
D	119	5·88	H	131	29·77	
Total . .		608	5·26		323	24 76

TABLE II

FREQUENCY OF MITOTIC ABNORMALITIES IN DIFFERENT TUMOURS AND IN DIFFERENT REGIONS OF THE SAME TUMOUR

(Koller, 1947a)

Skin			Cervix and corpus uteri (single biopsies)			Cervix No. 1 squamous carcinoma (multiple biopsies)		
Tumour	Cells in mitosis		Tumour	Cells in mitosis		Region	Cells in mitosis	
	Total number	Percentage abnormal		Total number	Percentage abnormal		Total normal	Percentage abnormal
Squamous cell carcinoma (cheek) .	135	2 23	Squamous cell carcinoma	132	3 03	A	51	3 89
Squamous cell carcinoma (forehead) . .	121	6 62	Squamous cell carcinoma	143	6 89	B	75	6 66
Basal cell carcinoma (forehead) .	141	9 22	Adenocarcinoma	150	14 00	C	71	8·46
Keratic squamous cell carcinoma (outer canthus) .	102	11·77	Squamous cell carcinoma	570	16 11	D	63	9 53
Squamous cell carcinoma (hand) .	153	16 99	Squamous cell carcinoma	150	16 67	E	97	14 44
Basal cell carcinoma (cheek) .	137	17·52	Squamous cell carcinoma	165	23 63	F	92	15 22
						G*	121	38 85

$x^2_{(5)} = 14\ 5127$ $x^2_{(5)} = 68\ 8522$ $x^2_{(6)} = 68\ 7677$

$P = < 0\ 001$ $P = < 0\ 001$ $P = < 0\ 001$

* This region showed extensive necrosis

early 1951, became polyploid by this process. According to Hsu (1954a) there are no diploid cells in this tumour (Fig. 58)
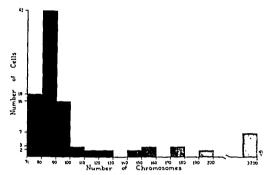
FIG. 58 —Frequency distribution of cells with different chromosome numbers in the *Hela* strain of human cervical carcinoma, grown *in vitro* (Hsu, 1954a)

The same type of variation in chromosome number found in normal cells has been observed in cancer cells cultivated *in vitro* (Goldschmidt and Fischer, 1929, Kemp, 1930; Levine, 1931; and others). The first important chromosome counts on solid tumour were made by Winge (1930) in 86 cells of tar induced papillomas in mice. The distribution of cells with varying chromosome number showed two maxima; one slightly less than the diploid number, probably 38 instead of 40, and another below the tetraploid, probably 68. Therefore it is not surprising that Winge accepted Boveri's theory that "malignant tumours are derived from one or several cells, which have an abnormal chromosome number." The author counted the chromosomes in the transplantable Walker carcinoma 256 of the rat and found that 58 is the most frequent chromosome number, that is, the tumour is hyper-diploid (2n = 42). Evidence was also obtained to show that the frequency of cells with 58 chromosomes has been fluctuating in the different transplant genera-tions of this carcinoma.

Cytological investigations have been carried out on tumour cells of the ascitic fluid of mice and rats. Reference is made to the articles by Hauschka and Levan (1951), Levan and Hauschka (1952), and Kaziwara (1954), in which a great variability in the chromosome number of various ascites tumours has been des-cribed and the cause of this variation discussed.

The data presented show that heteroploidy, that is cells with irregular chromo-some numbers, is common in human and animal tumours. Some animal tumours are known to consist of cell populations in which the cells with the normal diploid chromosome number are in the minority or *completely absent*. In view of this it

Mitotic abnormalities are also observed in animal tumours. Their incidence was studied in a number of sarcomas, produced in rats at the site of injection by aromatic mustards. It is shown in Table III that in the different primary tumours induced by the same mustard-compound, the type and the incidence of chromosome abnormalities differ greatly; in some tumours no abnormal mitoses were seen, and in others all types were represented with a high frequency. The most significant observation, however, was that most of the abnormalities were only temporary. They were not seen in the transplanted tumours derived from primary tumours rich in abnormally dividing cells.

TABLE III

CYTOLOGICAL CHARACTERISTICS OF NITROGEN MUSTARD INDUCED TUMOURS IN THE RAT

Compound	Tumour	No. of trans-plantation	Cytological features			Notes	Percentage of mitotic abnormalities
			Anaphase bridges	Frag-ments	Poly-ploidy		
CB 1048 N(CH₂CH₂Cl)₂	A	93 Gen	++ +	-	+	Permanent	68.5
	B	10	+	+	+	Temporary	5.6
	C	7	-	+	++	Temporary	5.0
	D	0	-	+	-	Not analysed	
	E	2	++	+	-	Temporary	6.0
	F	8	-	-	-	Normal	Nil
	G	3	+	+	+	Semi-permanent	12.6
CB 1047 N(CH₂CH₂Cl)₂	A	0	-	+	+		3.2
	B	0	-	-	++		9.5
	C	0	-	-	-		Nil
	D	7	+	-	+	Temporary	3.0
	E	4	+	++	+	Temporary	4.5
	F	0	-	+	-		7.5
CB 1044 CH₃—◯—N(CH₂CH₂Cl)₂	A	0	++	-	-	"Localized" chromosome stickiness	18.0
	B	0	-	+	+		Not analysed
	C	2	+	+	+	Temporary	Nil
	D	0	-	-	-		Nil
	E	2	-	-	+		7.0
CB 1074 N(CH₂CH₂Cl)₂	A	8	-	-	+	Temporary	5.7
	B	2	-	-	++		12.0
	C	0	-	+	-		7.0
CB 1075 CH₃ N(CH₂CH₂Cl)₂ Isomer M P 100°	A	0	-	-	+		3.6
	B	0	-	-	-		11.0
	C	0	+	+	++		7.0
	D	0	+	+	++	Treated with ACTH	Not analysed
	E	3	+	-	++	'Blister in tumour' contained CB1075	6.0
	F	0	-	+	-		10.0
	G	0	+	+	+	"Blisters"	22.5
	H	0	+	+	+		13.0
CB 1076 CH₃ N(CH₂CH Cl)₂ Isomer M P 83°	A	0	-	+	+		Not analysed
	B	0	-	-	+		3.2
	C	0	-	-	++	"Blisters"	7.5
	D	3	+	-	+	Semi-permanent	3.0
	E	0	-	-	+		5.2
	F	0	+	+	++		14.0

might be concluded that heteroploidy is the cause of malignant behaviour. However, before accepting this conclusion, it must be remembered that heteroploidy is also present in normal embryonic and adult tissues and that the growth of most human and animal tumours depends on cells with the normal chromosome number. Evidence has been obtained by the author which indicates that heteroploidy is due to mitotic abnormalities (Fig. 53d) the incidence of which can be influenced by the environment. The frequency of cells with different chromosome numbers has been compared in Yoshida sarcoma of the rat, growing as a subcutaneous solid tumour and as peritoneal ascites. It was found that heteroploidy is much greater in the ascites than in the solid tumour, and there is a direct relationship between the incidence of mitotic abnormalities and heteroploidy (see Table VI).

TABLE VI

VARIATION OF CHROMOSOME NUMBER IN YOSHIDA SARCOMA

Site of tumour	Chromosome numbers																			Total	Percentage of diploid cells	Percentage of abnormal normal mitosis
	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	50	56	80 Poly-ploid			
Ascitic fluid	3	1	2	1	6	1	2	—	2	1	10	2	8	2	3	1	1	1	2	48	20 8	8 8
Intraperitoneal adhesions	—	—	—	—	1	—	—	—	2	2	21	—	1	—	—	—	—	—	1	28	75 0	5 4
Subcutaneous (solid)	—	—	—	—	—	—	—	—	1 (?)		22	—	—	—	—	—	—	—	2	25	88 0	2 0

It is concluded, therefore, that heteroploidy is a secondary effect which follows malignant change and is not the cause of it.

Structural changes in the chromosomes of tumour cells

The essential feature of Boveri's chromosome theory is that the cancer cell has an abnormal chromosome complex or "constitution" (Chromosomenbestand). In the light of present knowledge the definition could include alteration in a single gene or block of genes within a restricted chromosome segment. In other words, the change would be a *structural* rather than numerical alteration in the chromosome complex. Because several of the structural changes, for example deficiency, deletion, translocation, inversion, can be recognized cytologically, there is the possibility that if these alterations are present in the chromosomes of cancer cells they may also be disclosed by cytological studies.

Measurement of changes

Quantitative comparison of the length of chromosomes in normal and malignant cells of man would be unprofitable because there is a graduation of size among the 23 autosomal chromosome pairs which makes it difficult, if not impossible, to identify alterations by direct measurement. There are other ways, however, by which changes may be detected; one is the comparison of the nucleolus-associated chromosome in the normal and the cancer cell. The cytological or chromomere

The variable frequency of abnormal mitoses observed at different points in space and time in the same tumour indicates that the mitotic disturbances are environmentally conditioned. Ludford (1930b) suggested that the irregularities seen in cancer cells are the result of the abnormal conditions present in tumour tissues. With a relatively poor vascular supply and the accumulation of the break-down products of metabolism and necrosis, there may be a sufficient degree of toxicity in the cellular environment to induce abnormalities. However, not all mitotic irregularities can be attributed to the unfavourable environment, because many of the abnormalities are present during the *in vitro* cultivation of tumour cells. The cause of chromosome irregularities may be in the metabolic organization of the malignant cell itself. There is evidence that the mitotic process is accelerated in cancer cells; the duration of prophase and post-metaphase stages is shorter than in mitosis of normal cells. The relative duration of the various stages was estimated from the frequencies of cells in prophase, metaphase and post-metaphase of mitosis shown in *Fig. 50.* The shortening of prophase stage is one of the most universal features of tumour mitoses (*see* Table IV). This effect may be another cause of many of the irregularities observed in dividing tumour cells (Koller, 1947b; Therman and Timonen, 1954).
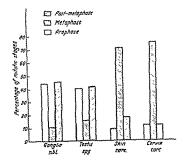
FIG. 50.—Histograms showing the relative frequencies of prophase, metaphase and post-metaphase stages in ganglial cells of a grasshopper (*Cortophaga*), in spermatogonial cells of testis and in tumour cells of man (Koller, 1947b).

It has been mentioned already that the same nitrogen mustard derivative (*see* Tables II and III), when injected subcutaneously into rats, produced tumours which differed in the frequency of their chromosome abnormalities. In some tumours they were completely absent. This finding provides further evidence that mitotic abnormalities are not an obligatory or universal feature of malignant

map of this chromosome is now known (Koller, 1953b). Another method is the analysis of chromosome structures in subdiploid malignant cells of human effusions in which unusual types can be more easily detected. The latter method has been followed by the author in a study of a pleural effusion of a patient with carcinoma of the breast. A very large chromosome with submedian centromere (17–18μ in length) was present in 23 per cent of cells. This particular "tumour-chromosome" is almost two-and-a-half times longer than the longest chromosome with a median centromere found in spleen cultures of a human embryo (Hsu, 1952). Furthermore, extremely small, dot-like chromosomes, which may be called "minutes," have also been seen in many tumour cells which are absent in the normal chromosome complex of man. These findings indicate that structural changes in tumour cell chromosomes do occur.

Animal tumours

The ascites cells of animal tumours are particularly suitable material in which to search for chromosomal structural changes. Bayreuther (1952) found in a hyperdiploid Ehrlich ascites tumour of the mouse, two exceptional chromosome types (designated as A and B). They could be identified easily by the median and sub-median position of the centromere, while in the normal chromosome complex of the mouse the centromere is situated at one end of the chromosome. In 75 per cent of tumour cells both A and B were present, but some cells contained A or B only. The two chromosomes are probably the result of duplication of chromosome segments. New chromosome types were also detected in other mouse ascites tumours studied by Hauschka and Levan (1951), and Fekete and Griffen (1954)

In Walker rat carcinoma 256 the author observed marked differences between the cancer cell chromosomes and those of the normal ordinary tissue cell. Most of the 42 (diploid) chromosomes of the rat are rod-shaped, having a centromere at the end, or nearly at the end of the chromosomes, while in the tumour studied by the author, the chromosomes were of three types; one with terminal, another with median, and the third with submedian centromeres (rod-shaped, v-shaped and j-shaped chromosomes). The rod-shaped chromosomes are the unchanged normal chromosomes, while the large v-shaped and j-shaped are new types, which persisted in transplantation (Fig. 49d). Similar findings were reported by Makino and Kano (1953) who studied ascites cells of three sarcomas of the rat. Makino (1952) has also analysed the chromosome constitution of Yoshida sarcoma cells, and found a similar situation.

The author observed another type of new chromosome in a tumour induced in the rat by subcutaneous injection of an aromatic mustard (Koller, 1953a); about 68·5 per cent of the dividing cells had chromosome bridges at anaphase (Fig. 59). The "bridge" configuration is known to be due to the presence of two centromeres in one chromosome (dicentric). In maize McClintock (1941) has shown, by the use of cytological and genetical methods, that bridges are formed by the fusion of two broken sister chromatids, which occurs during interphase when the broken chromosome divides into two sister chromatids (Fig. 57c and d). The configuration could persist almost automatically by the bridge-break-fusion cycle, and its presence has been observed in more than 150 transplant generations of tumours, though its frequency varied between 7 and 68 per cent. It is interesting that in tissue culture and in ascites the dicentric chromosome was absent.

TABLE IV

MITOTIC RATE AND RELATIVE FREQUENCIES OF MITOTIC STAGES IN
HUMAN CANCEROUS AND EMBRYONIC TISSUE

(After Iwanaga, 1954)

Tissue gastric mucosa	Mitotic rate as percentage	Frequency of stages as percentage			P/M Index
		Prophase	Metaphase	Anaphase	
Cancerous ..	2·7	37·3	55·2	7·5	<1
Foetal	2 1	66·5	27·0	6·5	>1

growth. Their presence in a tumour is a secondary phenomenon, that is they are the effect and not the cause of malignant transformation. They are also of secondary importance in tumour development because cells which are undergoing irregular mitosis usually degenerate and fail to contribute to the growth of the tumour.

Although mitotic irregularities are absent in some tumours, it might still be argued that abnormal mitosis need only occur once to alter the chromosome balance and produce a "primordial cancer cell." The possibility that such an event might have taken place previously or simultaneously with the malignant change is examined in the following section where the number and structure of chromosomes in normal and malignant cells are compared.

The chromosome number in the cells of normal and cancer tissue
Man

The chromosome number in human cells was first reported by Winiwarter (1912); he counted 48 and 47 chromosomes in the diploid (2n) oogonia and spermatogonia respectively. The cytological studies of Painter (1923), Evans and Swezy (1928), Koller (1937) and others showed that the diploid number is 48 in both sexes. Evans and Swezy (1928) also counted the chromosomes in embryonic tissues and found 48. Human fibroblasts have been grown *in vitro* by Kemp (1930); in most cells he found 48 chromosomes, but in a few there were small deviations from this number, involving 1 or 2 chromosomes. Greater deviations were observed in tissues of human embryos and embryonic membranes. Details of these earlier reports are given by Beatty (1954) By using a greatly improved technique Tjio and Levan (1956) recently found in 261 cells from 4 different human embryos only 46 chromosomes.

Timonen (1950) recorded the counts in 1,000 cells from normal human endometrium and found a variation from 4 to 104! In other publications written in collaboration with Timonen and Therman (1950a and 1950b) he reported a smaller range of variation in the endometrium, and in embryonic tissues. Manna (1954) found many hypodiploid and hyperdiploid cells in the endometrial mucosa (Fig. 51). A similar observation has been made by the author, who found considerable variation in chromosome numbers not only in the endometrium of different patients, but also in the endometrium of the same patient, taken at different stages of the menstrual cycle (Fig. 52). Hsu (1952) carried out karyological studies on
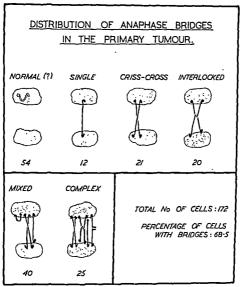
Fig 59 — Diagram illustrating the various types of chromosome bridges observed at anaphase of mitosis in a tumour of rat, induced by subcutaneous injection of an aromatic mustard (see Table III, CB1048, tumour A, and Koller, 1953a)

In the light of these observations it might be argued that the structural alterations responsible for the new chromosome types are the cause of the malignant transformation itself. In most human and animal tumours there is little evidence to suggest the presence of new chromosome types, yet structural alterations, which are confined to minute regions of the chromosome, could be present without being visible cytologically.

Changes caused by environment

While there is no evidence to show that structural chromosomal changes are the primary cause of cancer, there is evidence that such changes in the cell can be
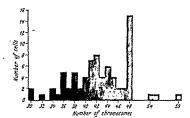
FIG. 51.—Histogram showing the frequency distribution of chromosome numbers in the endometrium (Manna, 1954).
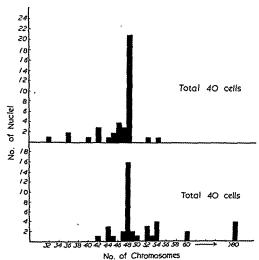
FIG. 52.—Histograms showing the variation in chromosome numbers in the endometrium of the same patient, analysed at interval of 59 days.

produced by the environment. The peculiar behaviour of the "dicentric" tumour chromosome, studied by the author, represents strong evidence that breakage and reunion of broken chromosomes, which underlie every structural change, can arise *de novo* in tumours. It is also of particular interest that the "cancer-chromosomes" referred to were found mostly in tumours, grown as ascites, in which the high incidence of mitotic abnormalities indicate environmental influences in action (Koller, 1956).

Somatic mutation and cancer

It has been shown that large, visible structural changes can occur within and between the chromosomes of tumour cells It is not known whether submicroscopic change, that is a mutation which is restricted to a gene or genes, also occurs in cells undergoing malignant transformation, and if it does what is the relationship between these two events.

One of the criteria of gene mutation is that the change is permanent: the mutated gene duplicates itself in the altered form and will be present as such in the cells descended from that in which the mutation has occurred. The other criterion of a mutation is that the altered gene is independent and can segregate from its normal, unchanged partner. This segregation can only occur in the gonads during gamete formation. While mutation in the gamete, that is *germinal mutation*, satisfies both of these requirements, mutation in other cells of the body, that is *somatic mutation*, can only be proved critically if the mosaic segment includes the gonad. The first well-defined theory of cancer as a somatic mutation was formulated by Bauer (1928), supported by Murray (1933), Boycott (1933), and Lockhart-Mummery (1934). This postulates that: (a) mutation takes place in the body cell; and (b) the mutation is the cause of the change from a normal cell into a cancer cell. In his original definition Bauer recognized three kinds of mutation: (1) alteration in a single gene, (2) in a group of genes, localized in a specific region of the chromosome; and (3) change in the chromosomes by means of deletion or duplication of part or whole chromosome. In the following part, only the first two types of mutation, namely mutation in a single gene, or in a group of genes will be considered in the light of recent developments in genetics; the third type of mutation has been discussed already in the previous part.

Single gene mutation

In the study of genetics much data have been accumulated which show that mutation of *single* genes in somatic tissues occurs in both plants and animals. The result of this event is a patch of tissue, genetically distinct from the rest of the individual. The size of the "sector" is determined by the stage of development in which the somatic mutation has occurred. Though the nature of the change can rarely be subjected to ordinary genetic tests, the occurrence of somatic mutation is an established fact and it has been demonstrated that its frequency in somatic cells can be increased in the same way and by the same agencies as that of germinal mutation, for example, x-rays, chemical mutagens and specific genes. An example of the latter type of somatic mutation is illustrated by the "dotted" gene of maize in chromosome 9, which induces the frequent mutation of a specific colour-

splenic explants of human embryo cultured *in vitro*; out of 124 cells, 73 per cent had 48 chromosomes, others contained 49, 47, 46, 45 and 44, and one cell showed 91 chromosomes.

In view of these reports, somatic inconsistency of chromosome number in human embryonic tissues and membranes must be accepted as a fact. The evidence for variation of chromosome number in the endometrium is also definite and cannot be disregarded. The fate of the chromosomally unbalanced cells in growth and differentiation is an important problem and is discussed later.

The chromosomes of human tumour cells have been counted by many cytologists, and while some found the number of 48 constant, others reported variation. Belling (1927) counted between 40 and 50 chromosomes in sarcoma cells, Evans and Swezy (1928) found only diploid cells, while Heiberg and Kemp (1929) noticed in a squamous-cell carcinoma of the cheek large tumour cells with tetraploid (96 : 4n) chromosome number. Similar observations were made by Levine (1930) who found giant cells with more than 200 chromosomes. The author analysed the behaviour and number of chromosomes in several human skin tumours and carcinomas of the cervix. In some, tetraploid and highly polyploid cells were frequent; in others the variation was restricted to around the diploid number. Cells with subdiploid chromosome sets were also observed. The dividing sub-diploid cells were usually lying adjacent to a dividing cell with a diploid or polyploid chromosome number (Fig. 53b) (Koller, 1947b). Manna (1955) counted the chromosomes of 1,011 nuclei from 29 patients with carcinoma of the cervix uteri (Table V), and found that a large number of cells have their chromosome number distributed within a short range and not necessarily around the diploid number.

TABLE V

SUMMARY OF THE DATA OF CHROMOSOME NUMBER IN TWENTY-NINE CASES OF HUMAN CANCEROUS CERVIX UTERI

(Manna, 1955)

No. of cases	Nuclei studied	Modal range	Nuclei within modal range as a percentage	Frequency peak (average)
9	317	36–47	79	42
2	83	43–54	69	48
5	131	49–60	69	58
9	395	61–72	45	68
4	85	73–84	55	76

The earliest observation, showing an unusually wide variation in chromosome number, was made by the author during the karyological analysis of human adenocarcinoma in the lower intestine (Koller, 1947a). The distribution of chromosome numbers is given in Fig. 54. While in this case the variation occurred in the subdiploid direction, Hsu (1954b) described a recurrent liposarcoma growing in tissue culture, having more chromosomes than the diploid number: the lowest number was 57 and the highest 346 (Fig. 55). Hsu also cultured free tumour cells of peritoneal and pleural effusions, and in metastatic melanoma he found that

controlling gene in chromosome 3. A large number of coloured patches of variable size are produced in the kernel. Another interesting and important group of somatic mutations are the so-called "bud mutants" in trees which can be propagated by grafting.

One of the main tenets of modern genetics is that genes determine the biochemical pattern of cells by controlling metabolic reactions, and that mutation in a gene alters the chemical environment of the cell on which depend normal development and cellular function. Since mutations are known to occur which affect the orderly balanced system of determining processes in somatic cells, a mutation might occur in a gene which controls the threshold of mitotic activity, and result in cancerous behaviour.

Gene-block mutation

The studies of the genetical basis of continuous variation disclosed the existence of genes, the effects of which are small, similar, cumulative and complementary to each other. These constitute a polygenic system, the individual members of which are referred to as polygenes (Mather, 1943). Polygenes form the genetical basis of quantitative characters such as weight, size, length, intensity of colour, and they presumably act by controlling the rate of biochemical synthesis involved in the determination of quantitative character complexes.

Analysis disclosed that polygenes are concentrated in the heterochromatin, the special chromosome regions of varying dimensions which under certain conditions fail to give the Feulgen-staining reaction. A close relationship has been demonstrated by cytochemical methods between heterochromatin and nucleic acid synthesis (Caspersson, 1939; Darlington, 1944) and it may be assumed that genotypic control is involved in cellular growth and multiplication. It can be expected therefore that mutation (genic or structural) in the heterochromatin, would affect polygenic complexes and cause disturbances in cellular behaviour. An instance has been reported in the millet (*Sorghum*), in which an increase in the number of supernumerary heterochromatic chromosomes led to extra mitoses in the pollen grain (Darlington and Thomas, 1941). The author has suggested that mutation affecting heterochromatin is an important event and that this change in the heterochromatin may also be associated with the origin of malignancy (Koller, 1943).

Thus modern genetic concepts lend support to the somatic mutation theory of Bauer, which in its early years met serious criticism. Some of the objections raised are attributable to a misunderstanding of the primary aim of the theory (for example, Blond, 1947). It is not claimed that the mutation theory explains the diverse and complex cellular reactions involved in malignant transformation, but it can be investigated. For this reason the somatic mutation theory has been revived following the discovery that chemical substances (for example the mustards, epoxides, and urethane) can induce germinal mutations. If it can be demonstrated that chemical carcinogens (for example polycyclic hydrocarbons) also induce germinal mutations this would represent a strong piece of evidence in favour of the somatic mutation theory. The relationship between chemical carcinogenesis and mutagenesis is the subject of the next section.

circulating 'heparin-like' substance, related to an increase in blood-clotting time, has been reported in acutely irradiated dogs[4]. The prolongation of clotting-time occurred before the platelet count was appreciably reduced, and had decreased to a normal value before platelet recovery could be demonstrated in the peripheral blood.

It has been suggested[5] that the increase in mast cells in the thymus of X-irradiated hamsters may augment the effects of the thrombocytopænia which occurs in these animals. It should be emphasized that, in twelve of our animals, abnormal bleeding into the gastro-intestinal tract was observed at a time when the bone marrow was apparently fully recovered and the platelet count could be expected to have returned to normal.

It would seem that the role of mast cells in the post-irradiation hæmorrhage syndrome deserves a careful study.

We wish to thank Prof. D. W. Smithers, in whose Department this work was carried out, and to the

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[1] Sparge, B., et al., J. Nat. Cancer Inst., 12, 615 (1951).
[2] Fisher, E. R., et al., Amer. J. Med. Sci., 223, 502 (1952). Jackson, A. H., and Hahn, P. F., Cancer, 8, 482 (1955). Botsford, T. H., et al., J. Amer. Med. Assoc., 151, 785 (1953).
[3] Asboe-Hansen, G., Cancer Res., 13, 587 (1953).
[4] Allen, J. G., and Jacobson, L. O., Science, 105, 383 (1947). Allen, J. G., et al., J. Exp. Med., 87, 71 (1948).
[5] Kelsal, M. A., and Crabb, E. C., Science, 115, 123 (1952)

THE RELIEF OF PAIN IN TERMINAL CANCER

CONTRARY to popular belief, cancer is not usually a painful disease; in fact the primary lesions are often so painless that they may grow to a considerable size before attention is directed to them. Sometimes, however, in the terminal stages pain may be severe, usually owing to the involvement of bone, or liver, or the infiltration of some part of the central nervous system.

It has truly been said that medicine is an art not a science and in no facet of medicine is this more apparent than in the management of the patient who is dying slowly from cancer. There is no rule of thumb by which analgesics can be chosen and no simple formula for assisting the patient's mental attitude to the pain and the disease. It is, however, the plain and unmistakable duty of every doctor, as soon as he is convinced that recovery is impossible, to devote all his art towards making the remaining days of his patient's life as pleasant and pain free as he can. Furthermore, under these conditions he need no longer be restricted in his choice of drugs for fear of subsequent addiction.

LOCAL TREATMENT

There are some general rules which may help to formulate one's plans. If pain is localized it may be best relieved by local treatment. This is particularly the case with single deposits in bone and also in the case of carcinoma of the bronchus. The pain from these lesions can often be much diminished or abolished by high-voltage therapy, although the course of the disease may not necessarily be affected. Another instance of the use of local treatment is the carrying out of cordotomy for the relief of intractable pain of nerve origin

ANALGESICS

When local treatment is not suitable or not effective, general analgesics must be exhibited. It is usually advisable to start with simple and non-habit-forming drugs, such as one of the many aspirin-codeine preparations, with or without the addition of phenacetin. If, as sometimes happens, patients find codeine very constipating, then the aspirin, phenacetin and caffeine combinations may be tried. These drugs I would regard as the outposts in the defence of the body from pain.

The next line of defence would consist of the more powerful drugs, such as methadone, usually known by its proprietary name of 'physeptone' (tablets or injection, 5 to 10 mg.), pethidine (tablets or injection, 25 to 100 mg.); or possibly the addition of sedative solution of opium B.P.C., 1949 (5 to 30 minims [0.3 to 1 ml.]) to the above-mentioned mixtures. Methadone and pethidine can be given by mouth without any side-effects, but sedative

Mutagenesis and carcinogenesis

Sacharov (1938) was the first to use a chemical carcinogen, 20-methylcholan-threne, on the eggs of fruit fly *Drosophila*; he found an increase in mutation rate The mutant genes studied were located in the sex chromosomes; they killed the male larvae. Auerbach (1939) found no mutagenic effect when she injected or fed larvae of *Drosophila* with another polycyclic hydrocarbon, 1 : 2 : 5 : 6 dibenzan-thracene. The experiments of Demerec and his colleagues (1949) in which adult flies were exposed to aerosols of various carcinogens, were also negative. Burdette (1950) douched the vagina of *Drosophila*, before copulation, but obtained no evidence of the mutagenic effect of polycyclic hydrocarbons. While most of their experiments yielded negative results, increased mutation rates were observed in *Neurospora* (Tatum, 1947) and in *Escherichia coli* (Latarjet, Buu-Hoi and Elias, 1950) after exposure to 20-methylcholanthrene.

Strong, in a series of reports (1926, 1940, 1945a, 1945b, 1945c, 1946, 1947, 1949a, 1949b) claimed to have induced mutations in mice, and in a large number of tumours, by the subcutaneous injection of methylcholanthrene. The occurrence of several changes in coat colour and pattern of pigmentation amongst the descend-ents of treated mice, led him to conclude that they represented germinal mutations. He also found differences in the frequency of methylcholanthrene-induced tumours in the various sub-lines which were derived from treated animals, and he assumed they were due to mutations in genes which determine the degree of susceptibility to tumours and which are located in different chromosomes. The increased mutation rate was calculated by Strong to be 1 in 400 mice as compared with the 1 in 26,250 non-treated animals. The importance of Strong's data could be evaluated by further investigation.

The mutagenic property of 1 : 2 : 5 : 6 dibenzanthracene in mice has been reported by Carr (1947, 1948). He injected the carcinogen into "mainly inbred" mice of dominant *lethal-yellow* genotype, and in the descendent generations in which "some in-breeding" had been permitted, 5 definite and 2 spurious mutations were found. In another experiment using CBA (non-inbred) strain, Carr observed one dominant mutation in 62 chromosomes analysed. As no genetic tests have been carried out to confirm whether the changes observed in the offspring of treated mice are due to gene mutation, the results require further comparative evidence.

It may be concluded that certain carcinogenic hydrocarbons can cause mutations in moulds and bacteria, and it is likely that they produce gene mutations in mice, in addition to tumours. The mutagenic and carcinogenic properties of numerous other chemical substances have been investigated in *Drosophila*, *Neurospora*, *Escherichia coli* for the genetic test and mice or rats for the carcinogen test. It was found that the aliphatic and aromatic nitrogen mustards, diepoxides, poly-ethyleneimines, dimethanesulphonoxyalkanes and urethane have mutagenic and carcinogenic activity. Reference is made to the papers of Auerbach (1949, 1951), Bird (1952), Woolley (1953), Boyland (1954) and Burdette (1955)

Amongst the physical mutagenic agents, ionizing and ultra-violet radiations are the most important and their effects have been studied in invertebrates and vertebrates. The mutagenic properties of x-rays were discovered by Muller (1927) and the mutagenic properties of ultra-violet radiation by Altenburg (1933). These

TABLE VII

MAMMARY TUMOUR INCIDENCE IN MICE

(Gruneberg 1952)

	Breeding females			Virgins		
Strain	n	Tumour incidence (percentage)	Mean tumour age in months	n	Tumour incidence (percentage)	Mean tumour age in months
C3H	1,888	91.4	8.6	150	91.4	11.1
	1,553	91.6	8.1	346	92.3	11.3
	200	75.0	10.7	—		
	605	92.3	10.0			
DBA	2,252	54.5	10.6			
	—	—	—	24	50.8	
	1,363	51.0	10.5	203	11.5	15.8
	3,241	64.5	10.6			
	50	76.3	12.7	197	72.1	13.4
A	788	83.2	11.5	244	4.5	17.5
	1,093	83.6	11.1	223	4.9	18.5
	730	72.0	12.1			
	348	73.0	9.8			—
BALBc	178	1.1	24-25	—	—	—
	502	1.4	19-29	—	—	—
C57Bl	240	0	—	—	—	—
	59	1.7	8.5	194	0	—
	568	0.5	18-29	113	0	—
	93	1.1	14.9	100	0	—

Cytoplasmic factor.—Reciprocal crosses between certain high-mammary and low-mammary cancer strains have disclosed that tumour incidence is high when the mother is from the high tumour strain and low when she comes from a low tumour strain. This difference indicates that a cytoplasmic factor is involved in the causation of mammary cancer in these strains. This cytoplasmic factor, called the "milk-agent" does not, however, appear to be an essential component in the development of mammary tumour, because there are strains which are free from this factor and yet develop breast cancer. The agent does not arise *de novo*, but must be introduced directly by breast feeding. The rôle of the milk-agent seems to be to increase the tumour incidence only. Thus the introduction of the agent from C3H strain has converted the very low cancer strain C (incidence 1·4 per cent in breeding females) into a high cancer strain (incidence 81·9 per cent) which is designated henceforth as C^+ (Andervont, 1941, 1945).

It has also been discovered that the propagation and transmission of the milk-agent are controlled by genes. This is similar to the case of *Paramecium* where the propagation of the plasmagene *kappa* is controlled by gene *K* in the nucleus. Data have been obtained also which suggest that the response of mammary gland tissue to the milk-agent is controlled by genetic factors.

This summary shows that several factors are involved in the development of breast cancer in mice and that of them the most important component is genetic.

radiations are known to produce cancer in animals and man (Furth, 1954; Blum, 1955).

Although many of those agents which produce cancer also cause gene mutations, it cannot be concluded that "all mutagens are carcinogens and all carcinogens are mutagens" (Strong, 1947); for example, formaldehyde is not a carcinogen, yet it is a mutagen in *Drosophila*; oestrogens are carcinogenic but no mutagenic activity has been recorded. In spite of these exceptions, which may be due to the fact that their mutagenic and carcinogenic activities were tested in widely different systems, that is in moulds and mice respectively, the striking correspondence between carcinogenic and mutagenic properties of the same chemicals cannot be dismissed as a coincidence. Muller (1954) attributed the carcinogenic effect of radiation to its property of producing gene mutations and showed that many chemicals can do likewise. He stated that the theory of somatic mutation of cancer has a high probability of being a fact.

Gene controlled tumour formation in Drosophila

About 24 different tumour strains are known in the fruit fly. The "growth" appears during the larval stage and becomes encapsulated in a sheath of melanin. During pupation the tumour undergoes histolysis with the rest of the larval tissues but with the exception of the melanin, which persists in the abdomen or thorax of the adult fly. Several of these tumours are due to multiple genes and others are due chiefly to the action of one gene. The penetrance, that is frequency of incidence, of this abnormality is influenced by the environment, and in one reported case cytoplasmic factors were involved (Makino and Kanehisa, 1954). Many of the tumours are associated with lethal genes. If the duration of the larval period is prolonged by the introduction of certain genes, for example "giants", the tumour can grow to a larger size and exert detrimental effects (Burdette, 1954; Oster, 1954).

The benign tumours of *Drosophila* originate from blood cells, some of which become melanized and accumulate in the distal haemocoele of the larva. Harnly (1955) studied a second chromosome recessive gene, *tu-c*, which was responsible for melanotic tumours with 96 per cent penetrance. He showed that the *tu-c* gene synthesizes a "carcinogen" which can be extracted and when injected into a non-tumour strain host produces a melanotic tumour. The histogenesis of gene-determined melanotic tumours has been analysed by Oftedal (1953), and Kaplan (1955), who found that the first phase of tumour development is an accumulation of spindle cells around the pericardial cells, followed by the enlargement of the tumour and an invasion of the fat-bodies. The terminal phase is melanization.

The only malignant tumour is that reported by Shatoury (1955); it has invasive tendencies and kills the larvae. I⸳ ⸳⸳⸳ ⸳⸳⸳ ⸳⸳⸳ ⸳⸳⸳ ⸳⸳⸳\
which is located in the sex chrom ⸳⸳ ⸳ ⸳ ⸳ ⸳ ⸳⸳ ⸳\
components of the lymph-node, ⸳⸳ ⸳ ⸳ ⸳⸳ ⸳ ⸳⸳\
at the end of 3rd instar stage. Many cells are released from the cancerous node; they invade various regions and organs such as the imaginal discs, nerve and fat-bodies, and the larvae usually die before pupation.

One of the most interesting phenomena is the "anti-neoplastic" activity of the third cell-type of the lymph node, called the platelets. These cells grow into giant bi-nucleate or tri-nucleate cells, which encapsulate the tumour and prevent its

Lung cancer

The strain differences in the incidence of lung tumours are shown in Table VIII. Spontaneous pulmonary tumours appear much later in life than mammary tumours, but when carcinogens are injected into mice susceptible to lung tumours, the latent period is reduced and the incidence and number of tumour nodules are increased. Thus in strain A mice, $1 : 2 : 5 : 6$-dibenzanthracene reduces the latent period from 18 months to 6 weeks, and increases the incidence from 90 to 100 per cent; the number of lung nodules is 75 per mouse. In the low-incidence C57L strain the same carcinogen induces pulmonary adenomas in 25 per cent of mice and the latent period is reduced from 18 to 12 months, the number of lung nodules being 0·27 per mouse.

TABLE VIII

INCIDENCE OF PULMONARY TUMOURS IN SOME INBRED STRAINS OF MICE

(Law, 1954)

Strain	Incidence of pulmonary tumours (percentage)	Other characteristics
A ..	80–90	Approximately 80% mammary cancer
C3H .	5–10	90% mammary cancer; 10–30% hepatomas
C57BL ..	<1	
C57L ..	<1	Less than 5% mammary cancer
BALB/c ..	20–30	
Swiss .	40–50	
C58 ..	<1	90% lymphocytic leukaemia
I . ..	10–20	Adenomatous lesions of stomach

The investigations of Heston (1940, 1941, 1942a, 1942b, 1954), Heston and Deringer (1947), clarified the genetical background of susceptibility to pulmonary tumour in mice. When the high incidence strain A was crossed with C57L, which is a resistant strain, mice of the F_1 hybrids and of the F_2 showed a different degree of susceptibility to pulmonary adenomas (Fig. 65). The statistical analysis of the data showed that at least 4 genes with additive effects form the genetic basis of susceptibility. Transplantation experiments indicate that the response to a carcinogen is determined by the genotype of the transplant and not by that of the host; that is the action of genes is localized within the pulmonary tissue.

Heston (1948b, 1954), Heston and Deringer (1947), found that the incidence of lung cancer is influenced by several genes: (1) *Lethal-yellow* (which controls pigmentation in the hair, causes obesity and kills the homozygous embryo) increases the incidence, while (2) *shaker-2*; (3) *hairless*; and (4) *flexed-tail* decrease the frequency of pulmonary tumours. These genes may affect the lung tissue directly, or act by controlling growth in general.

Leukaemia

This group of tumours in mice is of special interest because they are like those in man. C58 and AK strains have a 90 per cent incidence of leukaemia. Myeloid leukaemia is known to occur in the Rf strain and both lymphoid and myeloid

spread. The inhibitory action of giant cells is more than a mechanical result, for Shatoury found that the encapsulated spheroids and hexagons disintegrate while the free tumour cells become melanized.

Shatoury's studies suggest that during the early developmental stages of *Drosophila*, neoplastic and antagonistic tendencies are present, but are controlled by gene or genes, which probably constitute a polygenic system. Mutation in one of these genes can cause a switch towards one or the other tendency. For example, the gene *l-m* might increase the potency of tumour-promoting tendencies or reduce the capacity of the tumour-opposing ones.

In the offspring of *Drosophila* treated with diepoxybutane (a mutagen in the fruit fly and a carcinogen in mice and rats) about 25 per cent of the sex-linked lethal gene mutations are associated with melanotic tumours (Fahmy, personal communication). It is probable that the drug induced mutations in more than one locus, and one mutation may occur in the gene or in one of the genes, which control growth and differentiation.

The main importance of tumours in *Drosophila* is that their occurrence depends on one or several specific genes. Tumours with invasive tendencies described by Kaplan and Shatoury, are gene-determined developmental abnormalities, which in many respects are homologous to malignant growth of higher organisms.

The target theory of mutation and cancer

The genetical analysis of the radiation-induced mutations in *Drosophila* has shown a proportionality between the quantity of radiation received and the number of mutations produced. The quantitative relationship is interpreted in terms of the target theory. This assumes that gene mutation is caused within a specific volume, called the "target," by a single ionization, which provides the necessary energy (quantum) for molecular transformation (Timoféeff-Ressovsky, 1937, Muller, 1954).
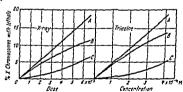
FIG. 60.—*Graphs illustrating the dose-response relationship between the various genetical changes in the chromosomes of Drosophila after x-ray and triazine (2:4:6:Tri-(ethyleneimino)-1:3:5-triazine). A total "lethal" mutational changes; B: lethals without visible chromosome rearrangements; C: lethals with rearrangements. (After Fahmy and Bird, 1953)*

A low radiation dose yields a low number of ionizations and thus the probability of the "target" being hit and a mutation occurring is low; conversely, a high dose has a high probability of causing mutation. When chemical mutagens were discovered, several investigations were carried out to determine the "dose-response"
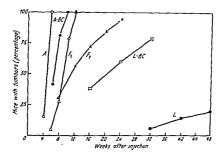
Fig 65 —Incidence of pulmonary tumours in the A and C57L (L) strains, and in the F_1, F_2 and backcross (A–BC, L–BC) hybrids, following intravenous injection of dibenzanthracene (Heston, 1942a).

leukaemias in the S and F strains, all with low incidence. Storrs-Little (StoLi) strain is a highly resistant line and with C58 was extensively used by MacDowell and his colleagues (1935, 1936, 1948) to study the genetic basis of susceptibility to leukaemia.

By crossing a single C58 high leukaemic male with three females of StoLi resistant strain, F_1 hybrids were produced and mated with low-leukaemic StoLi strain, and the first backcross mice were then mated with StoLi strain to obtain second backcross families. The incidence of leukaemia was then determined in 50 families as shown in Fig. 66. The distribution of leukaemias indicates that susceptibility to leukaemia depends on several genes. The results were complicated
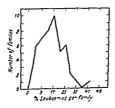
Fig. 66 —Graph showing the frequency distribution of second backcross families (C58 × StoLi) according to total incidence of leukaemia (Law, 1954)

relationship for chemically induced mutations. Fig. 60 illustrates the curves obtained by Fahmy and Bird (1953) for radiation and chemically-induced gene mutations There has been an increased interest in the dose-response characteristics for the production of tumours by carcinogens. It is hoped that, by extrapolating the data from dose-response experiments with *Drosophila*, the number of mutations or "events" required for the transformation of a normal into a cancer cell may be ascertained. If the target theory is applied to the study of carcinogenesis it follows there has been an acceptance of the assumption that the cell contains certain genes controlling growth and mitotic activity, and that the mutation must occur in these specific genes.

Charles and Luce-Clausen (1942) were the first to interpret the kinetics of papilloma-formation in benzpyrene-treated mice in terms of mutation rate. They found that the number of skin papillomas rises continuously from about five weeks after the first painting until about a month after the last painting of the carcinogen, and that the square root of the average number of papillomas per mouse formed a straight line when plotted against time after the first painting. Making various reasonable assumptions (namely that the mutation is the first step in malignant growth, that one cell is involved, that benzpyrene considerably increases the rate of mutation) they concluded that the linear relationship obtained when the square root of the number of papillomas is plotted against time as an expression of dose, indicates the occurrence of *two separate events* or mutations for the induction of a papilloma. A different conclusion was reached by Iversen and Arley (Iversen and Arley, 1950; Arley and Iversen, 1953) in their studies of the induction times of spontaneous tumours, and of those induced by polycyclic hydrocarbons, and Shope virus. They found a direct relationship between induction time and concentration of the carcinogen or virus, and inferred that the transformation of a normal to malignant cell is a *single event*. A similar observation was reported by Graffi (1953).

An analysis of dose-response relationship in the kinetics of pulmonary tumours was carried out by Heston and Schneiderman (1953) in strain A mice which were injected intravenously with 1 : 2 : 5 : 6 dibenzanthracene. They found a linear tumour-dose relationship (Fig. 61), which indicates a "single-action" within the cell as the basis of malignant change. If this single action is genic the change would be a dominant mutation.

The application of dose-response data in experimental carcinogenesis is potentially important for the testing of the mutation hypothesis of a malignant change. However, there are several prerequisites for their interpretation: (a) the tissue chosen should be stable; (b) the tissue chosen should be exempt from hormonal influences; (c) the carcinogen should have a short duration of action; (d) a single dose should be effective to produce tumour; (e) all tumours should be counted; and (f) no indirect effects should complicate the process of tumour induction. According to Wollman (1955) the data on which previous investigations relied fall short in many respects, particularly as to the total number of tumours or papillomas induced (for example, Shimkin and McClelland, 1949) and the complications due to indirect effects.

The experiment of Kaplan and Brown (1952) illustrates the difficulties which may be met with in the interpretation of dose-response curves. They produced thymomas in C57BL mice by x-rays, and found that the logarithm of the estimated average number of tumours per mouse versus logarithm dose of irradiation had a

by the numerous environmental variables, such as longevity, parturition, strain of nursing females, and sex.

Reciprocal backcrosses of hybrids between high and low leukaemic strains gave different results, indicating maternal influence (Table IX). MacDowell and Taylor (1948) found that in the crosses, the StoLi mother contributed a definite

TABLE IX

LEUKAEMIA IN THE STRAINS C58, STOLI AND CROSSES BETWEEN THEM

(Based on data of MacDowell and Richter, 1935)

	Leukaemia	No leukaemia	Total	Per cent leukaemia
C58	543	63	606	89 6
StoLi	4	302	306	1·3
C58 ♀ ♈ StoLi ♂ (A) ..	86	53	139	61·9
StoLi ♀ × C58 ♂ (B) ..	45	61	106	42 5
F₁ ♀ (from A) × StoLi ♂ ..	74	85	159	46 5
F₁ ♂ (from A) × StoLi ♀ ..	19	77	96	19·8

and specific resistance to leukaemia and this maternal resistance factor was more effective when the StoLi mother was old. On this basis experiments were designed in which the maternal resistance factor suppressed the development of leukaemia in susceptible strains. MacDowell's investigation showed that the maternal resistance factor can be transmitted through the milk, egg and the placenta.

At least two genes are known which are linked with susceptibility to leukaemia: dilution (*d*), and *flexed-tail* (*f*) are both plus-modifiers, that is they increase susceptibility. It is mentioned that *d* increases body size, while *f* produces anaemia in mice a few days after birth. Experiments also indicate that thymic tissue through some mechanism still unknown, influences the occurrence of leukaemia. The carcinogen-induced leukaemia incidence in CBA mice is reduced from 69·7 to 22·0 per cent after thymectomy. When the operation is followed by auto-plastic grafting of the thymus, the incidence is restored to 69·1 per cent (Law and Miller, 1950).

Reference is made to the claim of Gross (1954) that cell-free extract of AK leukaemia produced leukaemia in C3H mice, a strain free from this disease. Law, Dunn and Boyle (1955) were unable to confirm these findings in every detail.

The genetic basis of susceptibility to other tumours

The breeding experiments with mice with susceptibility or resistance to the three types of cancer already mentioned have shown that several genes are involved in determining the incidence of malignant growth. Results indicating the presence of multiple factors have also been obtained for other tumours; for example of the stomach, bone, bladder, and skin.

Stewart and Andervont (1938) found that old mice of strain I had a stomach lesion which begins to develop at 4 months of age and is accompanied by anaemia.
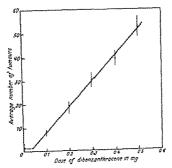
FIG. 61.—Graph showing a linear dose-response curve in the induction of pulmonary tumours by dibenzanthracene (*After* Heston and Schneiderman, 1953)

slope of 2; that is the average number of tumours per mouse is proportional to the square of the dose. It was discovered, however, that the bone-marrow under carcinogenic stimulation increases the tumour incidence in the thymus gland. The magnitude of this indirect effect cannot yet be measured and it is not possible to determine the number of induced changes or mutations necessary for the transition from a thymus cell to a malignant thymoma cell.

Advances in experimental procedure, as indicated by Wollman, will enable us to obtain more reliable dose-response data, which can be used in the study of carcinogenesis.

Age incidence of cancer and the mutation theory

The long latent period between carcinogenic stimulus and the appearance of cancer is considered a serious objection to the theory of somatic mutation of cancer. Bauer (1949) stated that the mutation responsible for the malignant change remains latent on the average 9 years for x-rays, 12 years for paraffin, 18 years for aniline and 40 years for solar radiation, but he gave no explanation of this Others recorded that the latent period for x-ray cancer varies between 2 and 49 years, irrespective of the dose (Petersen, 1954; Mitchell and Haybittle, 1955).

Blum (1950), using genetically-homogeneous material, uniform diet and treatment, reduced biological variability to a minimum in his experiments on ultraviolet radiation induced tumours in mice and found that the rate of tumour growth was not constant throughout the development time. If ultra-violet radiation induces mutations in cells, they should adopt immediately a new and constant

Though the lesion does not appear to be a malignant tumour, the animals have a short life. Grüneberg (1952) is of the opinion that this hyperplastic lesion represents a borderline case. Its incidence shows a great variation in crosses between strain I and various resistant strains; this suggests that several genes are involved in determining susceptibility. True adenocarcinoma of the gastric mucosa has been observed in NH mice. The stock is derived from inter-crossing CBA, IK and N strains (Strong, 1945a, b, c). When mice of NH line are injected with 20-methylcholanthrene, adenocarcinoma develops with high frequency, and the high susceptibility is transmitted to the offspring, designated as the NHO strain. Strong claimed that his observation indicates a two-fold action of the carcinogen (1) mutation; and (2) transformation: the first in a germinal gene for susceptibility to stomach carcinoma and the second as a somatic mutation in the gastric mucosa.

Bone tumours are rare in mice except in one strain developed from Simpson-strain by persistent selection and inbreeding. This is known as PM after Pybus and Miller (1938), who were responsible for establishing it. During five years of observation more than 300 osteogenic sarcomas were found in these mice. Reports now indicate that the susceptibility to bone tumours has been lost from the stock. From the PM strain a sub-line has been obtained in which uterine and cervical carcinomas occurred with high frequency.

Strain specific susceptibility to various cancers has been detected in mice by the use of carcinogens. C3H mice have the highest incidence of subcutaneous sarcoma at the site of injection, or at distant loci, after 20-methylcholanthrene treatment, while HR (hairless) mice have the highest frequency of squamous-cell carcinoma. The degree of susceptibility of various strains of mice to the action of carcinogens producing skin tumours is as follows: C3H > HR > C. > C† > C57L > C57B > DBA > I. The increase in the incidence of tumours above the basic level by the action of the carcinogen can only occur if the animals are susceptible, that is if the genetic factors are present. When they are absent, for example in resistant strains, the application of carcinogen does not produce tumours. An exception is leukaemia, because x-rays and chemical carcinogens readily induce the disease in mice which are known to be resistant, for example mice of strain C57B and strain A. Law (1954) put forward an explanation by assuming that the development of cancer is a threshold phenomenon. Some strains are far below the threshold and no tumour can develop after applying a carcinogen, while other strains are only slightly below the critical threshold and the carcinogen can raise these strains above the critical level and tumours appear.

In addition to the genes which are primarily responsible for the strain differences in respect of susceptibility to various cancers, many contributing non-genetic factors have been detected. Maternal influence, as an essential component of tumour incidence, has been mentioned. The age of mother, and frequency of parturition are other factors which can alter genetic determination. Changes in hormonal stimulation also influence the development of certain cancers. Thus, Woolley and Little (1945) were successful in producing tumours with a high frequency in the adrenals of mice of the CE strain after castration. The presence of specific genes, mostly determinants of morphological characters, for example *d* (*dilute*), *f* (*flexed-tail*), decrease the frequency of tumours in susceptible mice.

The combination of these factors with the multiple gene complex of susceptibility, make it difficult to clarify the Mendelian mechanism of the hereditary

rate of growth. Blum concluded that the rate of proliferation in certain cells is progressively accelerated by successive doses which explains the latent period and the sudden appearance of tumours, but is against the somatic mutation theory.

According to Berenblum and Shubik (1949) carcinogenesis is a two-stage process; the first is the *initiation*, the second is the process of *activation*. The initiating process represents a sudden and irreversible change in a small number of cells in the treated area, giving rise to isolated "latent" tumour cells, which remain morphologically like normal cells. These cells are further activated and subsequently become morphological tumours. The definition of the initiating process suggests a mutation-like change in the cell, and seems to support the somatic mutation theory. However, Berenblum and Shubik were unable to show that the mutagen mustard gas has any tumour initiating property and they question the applicability of the somatic mutation theory to carcinogenesis.

Muller (1951) postulated that many cancerous growths require not one but a series of mutations in a cell before the malignant change is completed. It is obvious that the time element constitutes an important factor in this process, and from the age incidence of cancer it would be possible to estimate the number of events or mutations which must occur in a cell before cancer appears.

Nordling (1953) analysed the frequency distribution of cancer according to age in man and found that within the age group 25-74 years, the logarithm of death rate increased in direct proportion to the logarithm of the age, but about six times more rapidly (Fig. 62). He suggested that this relationship can be explained if a cancer cell is the end-result of seven successive mutations. The hypothesis postulates that the probability of occurrence of each mutation remains constant throughout life! In spite of the fact that the high incidence of certain malignancies of children and women presents formidable deviation from the trend postulated by
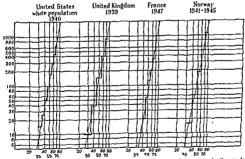

FIG. 62—Diagram drawn to double logarithmic scale showing the cancer death-rate (in the United Kingdom, the carcinoma death-rate) in males at different ages. Deaths per 100,000 males are shown on the vertical scale, age figures on the horizontal scale (Nordling, 1953.)

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transmission of a particular tumour. Furthermore this survey shows that in every instance in which genetically-conditioned susceptibility has been studied it has been found to be always influenced by environmental factors, some of which are known already, but many more have yet to be identified.

Genetics of tumour transplantation

The observation of fundamental importance, made by Loeb in 1902, was that a tumour which arose spontaneously in a Japanese waltzing mouse grew in all mice of the same strain but not in those of other strains. The role of the genetic constitution of the host in determining the fate of a tumour graft has been demonstrated by Little (1914), who found that the growth of a transplanted tumour depends on the presence of the same genetic constitution, that is genotype, in both donor and host mice. The laws of transplantation are summarized as follows:

(1) Tumours grow in and ultimately kill all animals of the strain of origin.

(2) Tumours fail to grow in unrelated strains.

(3) Tumours grow in F_1 animals one of whose parents is of the same strain as that in which the tumour arose.

(4) Tumours grow in a certain proportion of F_2 mice and backcross mice (F_1 × resistant parent) (*see* Table X).

(5) Tumours arising in F_1 will grow in all F_1 hybrids of the same strain, but will fail in either parent strains.

(6) Tumours arising in the F_2 will grow in all F_1 mice and a certain proportion of F_2 and backcross mice (Bittner, 1935).

(7) Certain tumours arising in F_2 may grow in all mice of one or the other of the parental strains (Bittner, 1935).

TABLE X

TRANSPLANTATION OF SPONTANEOUS MAMMARY ADENOCARCINOMA (14905a), OF "A" STRAIN ORIGIN

(*After Cloudman*, 1932)

Case	Host mice	Donor	Number of mice		Per cent positive
			Positive	Negative	
1	A	A	206	0	100
2	DBA	A	0	174	0
3	F_1	A × DBA	145	0	100
4	F_2	$F_1 × F_1$	67	154	30·3
5	A BC	$F_1 × A$	91	0	100
6	DBA BC	$F_1 × DBA$	6	81	6·9

The proportion of susceptible and non-susceptible mice in F_2 and backcross generations indicates the number of genetic factors involved, called the "histocompatibility or H-genes" (Snell, 1948). The lowest number, one-gene, was found for mammary carcinoma 17495a of strain A; the highest number is 12 and was found for mammary carcinoma 16189a of the same strain.

The host can be conditioned by injection of lyophilized tumour tissue, cell fractions or tissue antisera of the donor strain (Snell, 1954). This "enhancing effect" is probably immunological as demonstrated by Toolan (1955).

the hypothesis, it has the merit of explaining the latent period and the increased incidence of cancer with age.

The statistical method of Nordling was criticized by Stock (1953), who converted mortality rates into cohort rates for cancer of the stomach and estimated that the number of mutations required for malignant change is 5 instead of 7. Armitage and Doll (1954) found the regression coefficient for the incidence of cancer in different age groups varied from 4·97 to 6·48. According to them the observed
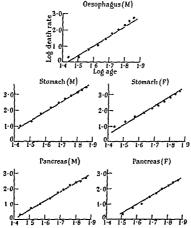
FIG. 63.—Change in mortality with age shown on a double logarithmic scale The death-rate per million persons plotted against the logarithm of the mid-point of the age group. The straight line through the points has been drawn arbitrarily to give the best fit, subject to the gradient being 6 to 7 (Armitage and Doll, 1954).

incidence of cancer of the oesophagus, stomach, colon, rectum and pancreas in males and of cancer of the stomach, colon, rectum and pancreas in women supports Nordling's hypothesis that 6-7 successive changes are necessary within the cell before cancer appears as a clinical entity (Fig. 63). For cancer of the lung, ovary and cervix in women, and cancer of the lung, bladder and prostate in men, the observation diverges from the theoretical regression curves either wholly or in part. The graphs shown in Fig. 64 illustrate significant deviations in cancer incidence for certain sites. The deviation from the six power may be due to the

Another phenomenon in tumour transplantation is the occasional change in the number of required histocompatibility genes. The classical case is the mammary adenocarcinoma *dbr-D*, studied by Strong (1926), which required 7 H-genes "Loss" or mutation in the H-genes resulted in the establishment of three tumour sub-lines: *dbr-Dm*, and *dbr-DB1* which needed 2, and *dbr-DBs* which needed only 1 H-gene. Several other instances are known in which mutation in histo-compatibility genes has occurred.

Sudden changes in transplantability have been found by transferring a tumour into an F_1 hybrid, a cross between the susceptible and resistant strains Barrett and Deringer (1950) described a three-fold increase in transplantability while Hauschka (1952) reported an increase as well as decrease in specificity in the transplantability of the DBA lymphoma and an increase in TA3 mammary adenocarcinoma. The experimental data are shown in Table XI.

TABLE XI

COMPARISON OF BACK-CROSS TAKE PERCENTAGES AFTER UNINTER-RUPTED PASSAGE OF MOUSE TUMOURS IN THE STRAINS OF ORIGIN AND AFTER ONE TRANSFER THROUGH F_1 BETWEEN SUSCEPTIBLE AND RESISTANT HOSTS

(Hauschka, 1952)

Tumour	Direct transfer % + in BC	Transfer via F_1 % + in BC	D/SE	Hypothetical shift in antigenic factors
C3HBA Barrett*	$7·8±1·6$	$24·0±3·2$	4 5	4 or 3 ⟶ 2
TA3	$28·0±3·2$	$11·2±3$ 1	3·7	2 ⟶ 3
DBATA	$24·0±2·1$	$11·6±3$ 2	3 3	2 ⟶ 3
DBATA	$22·0±2·1$	$34 0±4·7$	2 3	2 ⟶ ?
DBATS	$5·1±2·2$	$0 9±1·0$	1 8	4 or 5 ⟶ ?

* The data for C3HBA are those of Barrett and Deringer.

The detection of somatic mutations in transplantable tumours is simplified by the use of *isogenic* resistant strains (IR) which differ from the susceptible strains by a single histocompatibility gene. At present 70 of these strains exist and their number is increasing.

Tumours in animals and in plant hybrids

Hybridization between races, species, genera or strains, produces conditions other than those found in either parent. Metabolic processes are known to be controlled by genes, the rate and interaction of which will be affected to a varying degree by the altered genetic background of the hybrid (Wagner and Mitchell, 1955). The genetic disequilibrium may favour response to carcinogenic stimuli, and malignant growth develops, which rarely if ever occurs in the parental strain; for example tumours of the Harderian glands in hybrids of C3H × C57B cross. Table XII shows the variety of tumours obtained in F_1 reciprocal hybrid mice by Dickie (1954).

Evidence of an increased tendency to tumour-formation has been observed in the inter-specific hybrids between *Mus musculus* (C57B) and *Mus bactrianus*

fact that the incidence of ovary, cervical and prostatic cancers are under the influence of endocrine secretions and that the incidence of lung cancer is influenced by smoking habits in men.
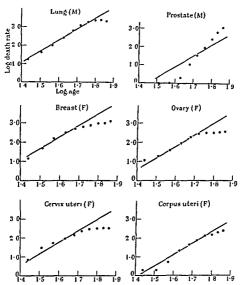
FIG 64—Graphs showing the change in mortality with age for certain cancers, in which the observation diverges from the theoretical regression curves either wholly (for example prostate, cervix and uterus) or in part (for example lung) (Armitage and Doll, 1954).

These statistical studies show that the hypothesis which postulates the occurrence of several mutations for malignant transformation has much evidence in its favour. For this reason we may be justified in recognizing "the multiple mutation theory of cancer" as a modified version of the somatic mutation theory. The multiple mutation theory might help to explain the significant deviations from the expected incidence, caused by divergences in social, economic and religious habits.

(986)

TABLE XII
TYPES OF TUMOURS FOUND IN F_1 RECIPROCAL HYBRID MICE
(*Dickie*, 1954)

CL × DBA .. 32 mice 2 lung tumours 6 lymphoid tumours	A × C3H .. 16 mice 14 lung tumours (87·50%) 7 mammary tumours (100%) 1 hepatoma
DBA × CE .. 29 mice 5 lung tumours 6 mammary tumours (42·85%) 1 papilloma 1 interstitial-cell tumour testis	C3H × A .. 20 mice 11 lung tumours (55·00%) 2 hepatomas 2 lymphoid tumours
DE × DBA . 34 mice 14 lung tumours (41 17%) 2 adenomas · Harderian gland 2 epidermoid carcinomas 3 fibrosarcomas 1 lymphatic leukaemia 2 papillomas 1 myoepithelioma sali- vary gland 3 hepatomas 1 giant-cell sarcoma	C × C3H 111 mice 7 lung tumours 3 ovarian tumours 6 uterine sarcomas 1 hepatoma 2 sarcomas

(Little, 1939). These species differ in size, rate of maturity and fecundity as measured by litter size. The hybrid mice showed a marked increase in tumour incidence, and the frequency of multiple tumours was about three times greater than in *Mus musculus*, one of the two parental species (Table XIII). Poll (1920) reported testicular tumours in species hybrids of birds (peacock and guinea fowl).

TABLE XIII
INCIDENCE OF TUMOURS IN TWO SPECIES OF MICE AND IN THEIR HYBRIDS
(*Little*, 1939)

	Total mice	Percentage with tumours	No. of tumours	Percentage of total mice with epithelial tumours	Percentage of total mice with non-epithelial tumours	Percentage of total mice with multiple tumours
Mus bactrianus	159	3 8	6	3·8	0 0	0 0
Mus musculus	877	14 4	137	1 6	13·2	7·9
F_1 Hybrids ..	121	45 5	72	13·2	39 66	23·6

The best analysed example of tumour production by hybridization is that of the melanotic tumours in fishes, studied by Gordon (1951, 1952). These tumours are determined by several genes necessary for the development and function of

macromelanophores, the pigment producing cells, and also by modifier genes which act as growth regulators. Their development is attributed to the genetic imbalance caused by hybridization. Melanotic tumours were observed in the *inter-generic* hybrids between *Platypoecilus maculatus* (platyfish) and *Xiphophorus helleri* (swordtail) and in the *inter-specific* hybrids between *Platypoecilus maculatus* and *P. conchianus*, which live 1,000 miles apart in Mexico. Gordon also found that certain *intra-specific* F_1 fishes may develop melanomas when the melanophore-genes and their modifiers were brought together by planned breeding.

Observations have been made on plant tumours which develop in certain inter-specific hybrids of tobacco (*Nicotiana*). Kostoff (1930) described 9 tumour-producing hybrids, and the number of known hybrid combinations is now about 26 (Kehr and Smith, 1954). The tumours, described more correctly as "atypical growths," develop usually in mature plants and can affect the roots, stems and less frequently leaves, but rarely kill the plant. Cytological studies of the tumourous growth have shown that, while mitotic abnormalities are absent in the parental species, their incidence is high in the *N. glauca* × *Langsdorffi* hybrid. In other hybrids irregular growth may occur and certain parts of the plant, usually the leaves, show sectorial mutation due either to gene mutation or to chromosome elimination. The cytogenetic studies carried out on various hybrids indicate that at least one whole set of chromosomes of one species must be present in the hybrid for predisposing tumour-formation.

Similar behaviour to that shown by the *Nicotiana* hybrids has been reported in certain inter-specific hybrids of *Datura* in which ovular tumours develop with high frequency (Satina, Rappaport and Blakeslee, 1951).

The occurrence of tumours in hybrids constitutes additional evidence showing the importance of the genetic background in the development of cancer. According to Kehr and Smith (1954), the growth regulating mechanism in hybrids has been disrupted by the combination of the two different genotypes and the "atypical growth" is attributed to the disturbed cellular metabolism.

Variation, adaptation and mutation in experimental tumours

Alterations in the structure, histological organization and behaviour of tumours have been observed by several investigators. Sarcomatous transformation of carcinoma is one of the best known examples, and occurs *in vitro* as well as *in vivo*. The cause of this may be the mixed epithelial cells and fibroblasts of the original tumour. This transformation would be the result of selective over-growth. The change, however, may also be interpreted by the plasmagene theory, according to which plasmagene under certain conditions can "infect" adjacent cells and change their behaviour (Billingham and Medawar, 1948).

Algire (1944) demonstrated that the morphological feature of tumours can be altered by selection. He produced a pigment-free tumour from melanoma S91 by selecting only "pale" tumour-grafts for transplantation. The loss of melanin and perhaps that of the pigment forming enzyme system is responsible for the development of "amelanotic" melanoma. This process closely resembles that described in *Paramecium*, in which the "killer" property can be lost. It has been shown that the cytoplasmic *kappa*, responsible for the secretion of the toxic substance, can be eliminated by accelerating cell division. Because the rate of multiplication of

subordinate to the cytoplasm in heteroploid tumour cells (Koller, 1947a). It also throws some light on the process of deviation from differentiation by assuming a competition between normal, unchanged and mutated plasmagenes. There is experimental evidence that this competition does exist in cells.

The author is of the opinion that the plasmagene theory represents a broader aspect of carcinogenesis and within its boundary all the other theories of cancer can be accommodated, and as Darlington stated "it enables us to take the cancer problem out of the lumber room of biology and use it as prop and buttress for the whole subject" (Darlington, 1948).

GENETICS AND EXPERIMENTAL TUMOURS

Inheritance of tumours in mice

The establishment of inbred strains in mice made it possible to investigate experimentally the respective rôles of genetic constitution (that is genotype) and of environment in the development of cancer. Differences between the various strains in the incidence and types of tumours developed are fundamentally genetic. Susceptibility to cancer in mice is inherited, but breeding tests have also disclosed the complexity of the genetic background. No tumour has yet been found in mice the occurrence of which is determined by a single gene. The studies indicate that many genes are involved in determining susceptibility, and that the genes have cumulative effects. In addition to the direct genic control, extra-nuclear factors, for example milk-agent in mammary cancer and the maternal resistance factor in leukaemia, can be involved also in the production of malignant tumours.

Some of the most important findings relating to the genetics of certain tumours in mice will now be discussed briefly. More extensive reviews have been published by Heston (1948a), Grüneberg (1952), Woolley (1953), Law (1954), and Shimkin (1955).

Mammary cancer

Several strains are known in which this tumour develops, but they differ in incidence and the age at onset. Table VII shows the frequency of mammary cancer in 5 pure strains of mice; the data were obtained in different experiments by several investigators, which accounts for the variation in tumour incidence within the same strain.

Extensive breeding tests, particularly those carried out by Bittner (1936, 1937, 1939, 1949, 1950) and Heston (1945, 1948a), have thrown light on the genetical and non-genetical factors in the development of breast cancer. Three factors influence its occurrence in mice: (1) genetic constitution, that is genes which condition susceptibility; (2) oestrogenic hormones; and (3) a cytoplasmic factor.

Genetic constitution.—The number of genes directly involved in determining susceptibility is not known, but breeding data indicate there must be several which are distributed in different chromosomes. The susceptibility-genes are closely linked with agouti, lethal-yellow, and brown coat colour, the genes of these characters being located in the fifth and eighth chromosomes.

Oestrogenic hormones.—The difference in the incidence of breast cancer in virgin and breeding females of the same strain indicates a strong hormonal influence, but the latter and the response of the ovary and mammary gland tissue to hormonal stimulation are under genic control.

kappa fails to keep pace with that of cell division, the number of *kappa* particles will gradually decrease in the daughter cells, which finally become completely free of it (Sonneborn, 1951).

Change can also affect physiological behaviour, as a result of which the tumour will show increased virulence, growth rate and altered specificity in transplantation. These changes could be brought about by (1) adaptation; (2) gene mutation; or (3) shifting the balance of histocompatibility genes through numerical changes in the chromosomes.

Physiological adaptation

The increased transplantability of a mammary adenocarcinoma, which was achieved by passing the tumour through F_1 hybrids, has been interpreted by Barrett and Deringer (1950) as an example of physiological adaptation. The process is similar to that described by Spiegelman (1951) who analysed the long-term adaptation to galactose and melibiose fermentation in yeast and found that contact with substrate induced modification for the formation of enzyme, which was transmitted indefinitely in the presence of the substrate. Increased transplantability could also be the result of an unequal production of certain intracellular antigens as suggested by Gorer (1948). In both instances the tumour genotype has not changed, and therefore the different behaviour may be a physio-logical adaptation.

Gene mutation

Mutation in the histocompatibility genes of tumours has been reported by Strong (1926), Snell (1948), Hauschka (1953), and several others. Borges and Kvedar (1952) found that mutation of the H-gene can also occur in the host. Owing to gene mutation, it is likely that the tissue of tumours contains cell variants with different histocompatibility gene complexes. As the cells with the mutant H-genes can be sorted out by selection, they form the source of new growth-potentialities in the tumour.

Numerical changes in the chromosomes

Alteration in transplantability of tumours can also be brought about by numerical changes in the chromosome set. Such a change represents a drastic re-arrangement of genic equilibrium and would affect the histocompatibility genes. Hauschka (1953), and Hauschka and Levan (1951, 1953) analysed the relationship between immuno-genetical properties and chromosome numbers of tumours and concluded that "tumours with diploid chromosome number are completely strain specific, while tumours with tetraploid or near tetraploid constitution exhibit various degrees of genetic indifference with regard to their host-requirements."

The Krebs-2, Ehrlich, Sarcoma 180 and 37, S91 Melanoma and Walker rat carcinoma are non-strain specific and all have heteroploid chromosome numbers. It is not improbable that they became freely transplantable because of the chromosomal duplications, which shift the balance of the histocompatibility gene complex.

In view of the fact that gene mutation and numerical change in chromosome composition occur in tumours, the cell population of a malignant growth is a

solution of opium may cause constipation owing to the opium content (this can be corrected by a mild laxative) and, on occasion, in a morphine-sensitive person, it may cause vomiting. There is a vast range of proprietary analgesics which fall somewhere between the two groups already mentioned in their strength of action and some doctors will have a preference for one or other of these, such as 'anadin' or 'edrisal'.

MORPHINE AND ITS DERIVATIVES

The next line of defence is the main and most solid line and is soundly based on the pain-relieving qualities of morphine and its derivatives. Morphine itself (1/6 to ½ grain [10 to 30 mg.]), 'omnopon' (1/6 to 1/3 grain [10 to 20 mg.]), dihydromorphinone hydrochloride, B.P.C. 1949 ('dilaudid') (1/32 to 1/16 grain [2 to 4 mg.]) and finally diamorphine hydrochloride (heroin) (1/12 to 1/3 grain [5 to 20 mg.]), all most effectively given by subcutaneous injection, are those in common use. The chief disadvantage of morphine is that in from 5 to 10 per cent. of patients severe nausea or vomiting occurs and cannot always be controlled by such simple additions as 'avomine', 25 mg., or pyridoxine, 10 to 100 mg. Patients rarely suffer from vomiting following 'dilaudid' and hardly ever after heroin. If there is very severe pain, morphine or 'dilaudid' may be more effective; if severe discomfort and depression or anxiety, then heroin is the drug of choice. In the case of all these morphine-group drugs constipation is likely to occur and should be guarded against by giving mild laxatives, unless they themselves are contraindicated by the nature of the lesion.

If pain is very intractable and all else has failed a reinforced morphine or heroin effect may be achieved by combining these drugs with amiphenazole ('daptazole'), which diminishes the depressant action without altering the analgesia; doses up to 1 to 2 grains (60 to 120 mg.) of morphine, ½ to 1 grain (30 to 60 mg.) of heroin may then be given. Alternatively, if the patient has more general discomfort and depression than pain, what is known at the Royal Marsden Hospital as haustus 'E' ('E' for euphoria) is used. This consists of:—

Morphine hydrochloride ...	¼ grain (16 mg.)
Cocaine hydrochloride.. ...	⅙ grain (10 mg.)
Gin......................	60 minims (4 ml.)
Honey	60 minims (4 ml.)
Chloroform water........ to	½ fluid ounce (15 ml.)

The prescribing of gin on an E.C. 10 prescription form is liable to be frowned upon by the Ministry of Health. As a way of getting round this difficulty, C. A. Clarke (*The Practitioner*, 1957, **178**, 38) recommends prescribing 'gin' as follows:—

'Rectified spirit 120 minims (8 ml.) with tincture of capsicum ½ minim (0.03 ml.)'

The initial dose of haustus 'E' is ¼ to ½ fluid ounce (7 to 15 ml.) and this usually relieves discomfort and gives the patient a pleasant drowsy feeling.

Finally, there can be no doubt that with incurable cancer in the terminal stage the patient should be kept pain free, even if this means keeping him practically unconscious. There can be no excuse for allowing patients to suffer unnecessarily once the diagnosis has been made and the inevitability of an early death is apparent.

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mosaic of numerous cell variants (Klein, 1955). The selection pressure exerted by the host, and also by drugs or x-rays, can lead to a gradual, or occasionally to sudden, transformation of tumours. This transformation has been produced by exposure of leukaemic cells to antimetabolites. According to Law (1954) the "mutation" to resistance or dependence occurs spontaneously in the leukaemic cell population; the role of antimetabolites is that of a selective agent only

Those few examples are sufficient to show that not only tumour-susceptibility, but the character and behaviour which malignant growth displays during development, are also regulated by genetic factors.

HEREDITY AND CANCER IN MAN

Introduction

The possibility that cancer in man may have a genetic basis is suggested by the publication of a number of pedigrees showing the high incidence of particular forms of the disease in members of one family. One of the most famous pedigrees is that of the family "G" which has been analysed by Warthin (1913, 1925) (Fig 67). The incidence of cancer amongst members, who reached the "cancer-age" is 31·8 per cent and Warthin concluded that a recessive familial susceptibility to
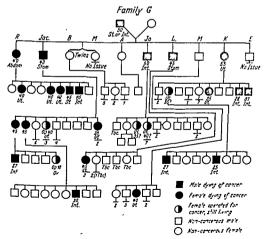

FIG. 67.—The pedigree of three generations in the family "G" of Warthin The site of cancer and age at death is indicated (*After Broebeck*, 1949)

TABLE XIX
DISTRIBUTION OF TUMOURS IN TWINS (IN PERCENTAGE)
(Macklin, 1940)

Type and site of tumour	Monozygotic		Dizygotic	
	Concordance	Discordance	Concordance	Discordance
Any type, all sites ..	61 3	38 7	44 4	56 6
Specific type, same site ..	55 0	42 0	24 2	76 8

Gorer (1953) analysed the occurrence of gastric carcinoma in pedigrees of twins collected by various authors. The data of Table XX show that in 5 of 19 monozygotic pairs both were affected, in 5 the tumour was at the same site. In the dizygotic twins only one case of concordance was found. The differences between the two series of twins suggests that stomach cancer has a hereditary basis. On the other hand, a similar analysis of twins with mammary carcinoma has shown more concordance in dizygotic then in monozygotic twins, and Gorer drew the conclusion that the role of a genetic component in the causation of breast cancer is secondary.

TABLE XX
CANCER OF THE STOMACH IN TWINS (UNSELECTED MATERIAL ONLY)
(Gorer, 1953)

Authors	Monozygotic pairs			
			Concordant	
	No. of pairs	Discordant	Same tumour	Different tumour
von Verschuer and Kober (1940) ..	6	4*	2	—
Busk and his colleagues (1948) ..	6	5	—	1*
Others	7	3	3	1*
	19	12	5	2*
	Dizygotic pairs			
von Verschuer and Kober (1940) ..	17	13	—	4
Busk and his colleagues (1948) ..	8	6	—	1
Others	9	4	1	4†
	34	23	1	9

* These tumours were both in the large intestine   † One with tumour of large intestine
According to a recent communication from Verschuer, 2 more monozygotic pairs developed cancer of the stomach.

Genetics of pre-cancerous conditions

Multiple polyposis of the intestine

Multiple polyposis of the intestine usually leads to carcinoma of the lower digestive tract. Studies of family histories indicate that polyposis is genetically

cancer is present, affecting the gastro-intestinal tract of the males and primarily the uterus of the females. Waaler (1931) studied familial histories of cancer patients in Holland and found that the incidence is higher for male relatives of patients than it is for the husbands of patients, or for the general population; this indicates a hereditary susceptibility. An impressive number of pedigree charts have been compiled and analysed by Lentz (1947) and Schintz, Cocchi and Neuhaus (1948). Three pedigrees are illustrated in Figs. 68*a* and *b*, 69, taken from Lentz' collection.

u = uterine carc.
g = gastric "
p = pancreatic "

(a)

bst = breast carc
c = cervix "
g = gastric "
lg = lung "
o = ovarian "

(b)
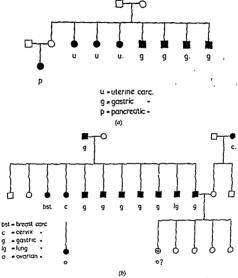
Fig. 68 —Pedigrees showing incidence of various cancers. (*a*) carcinoma of the uterus and stomach, (*b*) mixed types. (*After Lentz, 1947.*)

Schintz's pedigrees contained over 25,000 individuals and were followed through six or seven generations. The authors found that the frequency of carcinomas is increased amongst close relatives (sisters, brothers, and parents) of the probands or propositi with carcinoma. Contrary to expectation the incidence in relatives of patients with sarcomas was lower than in the general population.

conditioned, being inherited as a dominant character. Dukes (1930), Lockhart-Mummery (1934) and Gardner (1951) recorded pedigrees in which several members of family groups were affected with the disease (Fig. 73).

☐ Cancer of rectum or colon
Polyposis not proven

■ Cancer of rectum or colon
and polyposis

Fig. 73.—Pedigree of multiple polyposis of the intestine. (*After Dukes, 1952.*)

It has been found that several patients with carcinoma of the lower digestive tract had also skin tumours. There are records of families in whi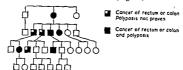ch not only surface tumours but osteoma, localized to the mandible, maxillae and other cranial bones, were also diagnosed in the presence of polyposis (Gardner and Richards, 1953). It is possible that the cancerous lesions of different sites are pleiotropic manifestations of the same gene. If, however, there are several genes involved, a very close linkage of these genes must be postulated. Neel (1954) estimated the frequency of multiple polyposis in the State of Michigan, United States of America to be 1 in 29,000 individuals, the frequency being about 15 times less than was calculated for neurofibromatosis.

Xeroderma pigmentosum

Another pre-cancerous condition is xeroderma pigmentosum, characterized by dryness, pigmentation and ulceration of the skin, which is followed by the development of epithelioma or basal-cell carcinoma. This condition is attributed to hypersensitivity of the skin, particularly that of the basal cell layer, to sunlight. Cockayne (1933) by analysing the family histories of patients, concluded that xeroderma depends on a recessive autosomal gene. In the 43 family histories he studied more than 30 per cent of the patients came from first cousin marriages. Haldane (1936), in his search for incomplete sex-linkage in human pedigrees, analysed 82 family histories collected from various sources, and by employing new statistical methods concluded that the gene xeroderma is incompletely sex-linked. The present author studied the occurrence of xeroderma pigmentosum in three families; in one family several members were affected (Fig. 74) (Koller, 1948). In this particular family there were unusual features—the late manifestation of the disease and an unexpected segregation of the condition among the sexes. These facts led to the conclusion that the gene responsible for the mild form of Xeroderma may have been transferred to another region of the sex chromosome, and as a result it shows a position effect. The other possibility is that another independent autosomal gene is responsible for the mild condition in this family.

Racial incidence of cancer

In mice there are strains in which the incidence of certain types of cancer is very high. It has been demonstrated by a large number of workers that the

In the study of family pedigrees with cancer, however, there are many factors to consider, many of which are not fully understood. For example, it must be remembered that cancer is a fairly common disease in the general population, and

bst. = breast carc.
liv. = liver "
g = gastric "
u. = uterus "
(bm) = brain metastasis
bl = bladder carc.
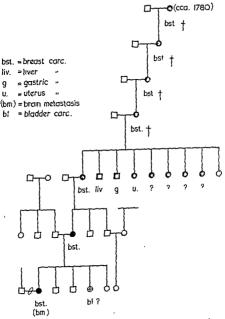
FIG. 69.—Pedigree showing the occurrence of breast cancer in 7 generations.
(After Lentz, 1947.)

therefore it may sometimes be expected to occur in several members of one family by chance alone. There is also the difficulty of diagnosis, and also an error cannot be avoided when the data are obtained from different sources. Most of the pedigrees are incomplete, as the cause of death of deceased relatives cannot be known with certainty. The shortcomings of pedigree analysis are further
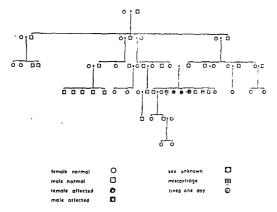
FIG. 74 —The pedigree of a family in which xeroderma appeared in a mild form
(Koller, 1948)

difference between strains is determined by the particular genotype. The question
naturally arises whether a similar situation exists in man. Comparison of the
incidence of the same cancer in various racial groups has revealed some interesting
facts, which initially seemed to suggest that genetically determined racial differences
for certain cancers may exist.

Primary carcinoma of the liver

The best illustration is that of the primary carcinoma of the liver. It is uncommon
in Europeans, but in Africa, particularly in the Bantu tribes, it is the most common
variety and has been referred to as a "racial" cancer, implying that in the African
negroes there is a hereditary tendency to develop hepatic cancer. An objective study
of the primary liver carcinoma in the Africans, however, has revealed that it is
environmentally conditioned and dietary deficiencies are mostly responsible for
the high occurrence. Davies (1955) found that in 3,573 autopsy cases, 17 per cent
of liver cirrhosis was associated with carcinoma. Cirrhosis represents an area of
chronic tissue damage and inflammation which are predisposing factors for
hepatoma and brought about by protein and fat deficiencies in the diet during
childhood. When these dietary deficiencies are eliminated the incidence of primary
liver cancer is expected to decrease. This has happened in the case of Negro
migrants of the United States, and led Steiner to state that this should clearly
dispel the widely held belief in a racial hereditary predisposition (Steiner, 1954).

exaggerated by the small size of the human family, the long life span of generations, and the late onset of the disease. A large and unbiased control group is almost impossible to establish in practice, which makes any comparison unreliable (Macklin, 1954). When these difficulties are considered it is obvious that the study of cancer in families is of limited value and generalizations must be made with caution (Neel, 1955).

A brief summary is given of some investigations on the hereditary aspect of various types of human cancer. A good critical review was made by Gorer (1953).

Cancer of the breast

This form of cancer is easily recognized and its presence in close relations is usually recorded in the family history of patients. In 1866 Broca made a family record in which 15 out of the 38 female members died from disease.

TABLE XIV

THE INCIDENCE OF CANCER IN NEAR RELATIVES OF PATIENTS WITH BREAST CANCER

(After Jacobsen, 1946)

	Number		Cancer of all sites		Breast cancer	
	Patients	*Controls*	*Patients*	*Controls*	*Patients*	*Controls*
			Per cent	Per cent		
Mothers	200	200	55=27·5	19=9·5	21	2
Fathers	199	199	40=20 1	19=9·5	1	0
Sisters	381	433	30= 7·9	8=1 8	13	2
Brothers	377	389	16= 4·2	4=1·0	0	0

The first genetical study was made by Jacobsen (1946), on the near relatives of 200 cases of mammary carcinoma (Table XIV). While cancer was present in 77 per cent of these 200 families he found the disease only in 29 per cent of 200 control families. Jacobsen concluded that a hereditary predisposition is the chief factor in the development of breast cancer. Kemp (1948) stated that the pre-disposition is non-specific and the localization of the tumour is determined by other genetical factors and the environment may also play a part in the development.

Other extensive analyses have been carried out by Penrose, Mackenzie and Karn (1948) on 510 patients and by Smithers and his colleagues (1952) on 704 patients. They found an increased incidence of breast cancer in sisters and mothers, and more cases in maternal grandmothers and aunts than in paternal grandmothers and aunts (Table XV). According to Penrose the evidence suggests the existence of a "specific genetical agent" which may be cytoplasmic. Several remarkable pedigrees showing the high incidence of breast cancer have been reported by Smithers (1948) (Fig. 70). While he found a significantly higher death rate from cancer of the breast in families of patients with the disease, there was no evidence that cancer tends to develop earlier in patients whose relations are known to have suffered from the disease, as was concluded by Jacobsen.

Penile cancer

Penile cancer is known to be very frequent in certain parts of China (15 per cent of all cancers) while it is almost totally absent in the Jews. Similar differences have been found between the Hindu and the Muslim populations of India; penile cancer is about 20 times more frequent in the former racial group. It was shown that circumcision in early life is the chief factor responsible for the lower incidence of penile cancer in Jews and Hindus. The difference is not genetical but cultural.

Skin cancer

Racial differences have been observed in skin cancer; for example negroids and other races with pigmented skin have significantly less cutaneous cancer than the white races. The difference has a genetic basis but the genetic control is indirect and exercised by genes which determine the degree of pigmentation in the skin. The albino negro suffers from skin cancer just as much as white persons (Shaplin, Keen and Murray, 1953).

Cancer incidence in migrants and sedentes

One of the most valuable methods to reveal racial differences is the comparison of cancer incidence in migrants and sedentes. Steiner (1954) carried out an analysis of 35,293 autopsies at the Los Angeles County Hospital in California. His material was drawn from 5 racial groups and it demonstrated differences, perhaps genetically determined, in the organ distribution of cancer. He found extremely low incidences of prostatic cancer, mammary adenocarcinoma and cancer of the oesophagus in the Mexicans. However, he did not find any example of cancer occurring in one race only. His studies showed that group differences in cancer incidence exist which are caused by racial-hereditary, geographical, social, economic, occupational and cultural factors. His investigation demonstrates the value of analysis of cancer incidence in racial groups, which lies in the fact that cancer predisposing environmental conditions may be detected and preventive measures may be introduced.

Cancer risk

In the previous part a few examples showing high familial incidence of certain types of cancer and the conclusions drawn by various investigators have been presented. Though many of the studies are incomplete or imperfect, the high incidence of the disease in related members within groups cannot be attributed to chance only and the data suggest a parental influence in the causation of certain cancers. The analysis of family pedigrees, however, has failed to establish the method of transmission. The mode of inheritance expressed in Mendelian terms is known only for a few and very rare malignant and related diseases, for example retinoblastoma, and neurofibromatosis, polyposis and exostoses. Before the exact mode of inheritance can be determined, it is necessary to know the carriers of the genes for cancer susceptibility. Cancer manifests itself in different ages, and in order to obtain such information an age correction must be made for relatives of the cancer patient, who have not yet reached the cancer age. The risk involved for a person to die of cancer must also be determined for each individual category of relative such as mother, father, sister. This analysis has been made by Danish workers for cancer of the breast and uterus. Table XXI shows the cancer hazard
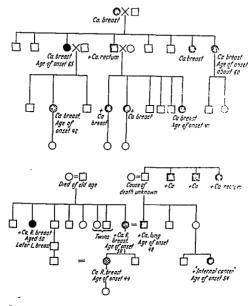
FIG. 70 —Two family histories showing a high incidence of breast cancer in the female members (Smithers, 1948).

Macklin (1952), by employing a more elaborate statistical analysis, found that cancer of the breast is 2–7 times more frequent in the different relationships of the breast cancer relatives than it is in the same relationships in control populations. It was also more common in deceased relatives of the cancer patients than in the deceased relatives of the controls. The incidence of breast cancer among the paternal and maternal grandmother and aunts of the cancer patient was the same, which seems to reduce the significance of environmental influences and the "milk agent." Macklin (1954) discovered that in male relatives of breast cancer patients, prostatic cancer is more common than in the control population. This finding is

among relatives of patients with uterine cancer (Broebeck, 1949). The calculations were based on various assumptions and further studies are needed to substantiate them.

TABLE XXI

ESTIMATE OF THE CANCER RISK AMONG RELATIVES OF PATIENTS WITH UTERINE CANCER (EXPRESSED AS A PERCENTAGE)

(Broebeck, 1949)

Material	Relatives	Cancer of all sites	Relatives	Cancer of all sites	Uterine cancer
Danish population	—	29 2	—	29 ·	2 9
Cancer of the corpus	Father	38 0	Mother	40 ·	9 6
	Brother	34 6	Sister	46 5	9 4
	Maternal grandfathers	25 2	Maternal grandmothers	24 3	2 2
	Paternal grandfathers	46 7	Paternal grandmothers	37 3	6 5
	Maternal uncles	38 2	Maternal aunts	38 7	4 4
	Paternal uncles	38 7	Paternal aunts	28 1	4 8

Most genetic variability in the human species is polygenic Evidence is accumulating that predisposition to susceptibility for pathological conditions depends on several genetic factors, many of which may act as modifiers It is likely that in some cases, and particularly in cancer, the environmental influences (for example exposure to various carcinogenic stimuli) play the important, perhaps decisive role, in the activation, while the genetic control affects only the development and manifestation of the disease. What must be determined is the relative importance of environmental and hereditary influences in the causation of cancer, and how far can the latter be modified by controllable environmental influence This should be considered when an attempt is made to interpret the discordant occurrence of cancer in twins. As an example, the low incidence of breast cancer in monozygotic twins may be mentioned. The data of Habs (1939), and von Verschuer and Kober (1940) may be interpreted as evidence against the presence of a hereditary component in the aetiology of mammary carcinoma. On the other hand, a different conclusion may be drawn, namely that the high proportion of monozygotic twins, in which only one member was affected with cancer of the breast, is evidence that genetic factors can be successfully suppressed by environmental influences. Studies of the latter should yield valuable information concerning the problem of cancer prevention.

The absence or rarity of penile cancer in the circumcised Jews and Hindus, on the one hand, and in the Japanese, who do not follow the practice of circumcision, is another example showing that environmental influences may be of primary importance in one racial group, whereas in another the genetical control is of greater importance.

The significant differences in the frequency of certain cancers between the male and female sex are usually interpreted as evidence against a hereditary basis. Many examples of "sex-limited" characters are known in classical genetics, and the sex difference of cancer at a particular site (for example oesophagus, lung, stomach) is not sufficient to deny the presence and importance of genetic factors.

Statistical investigation of the occurrence of *multiple primary carcinomas* represents another important method in the study of the hereditary aspect of

of great interest in view of the relationship of sex hormones to cancer of the breast and prostate.

Though criticism may be raised against the pedigree analysis of some workers, the evidence indicates at least in certain families the presence of a significant genetic component in the causation of breast cancer.

TABLE XV

HEREDITY IN HUMAN BREAST CANCER

Comparison of data collected by Penrose, Mackenzie, and Karn (1948) with that from the Royal Cancer Hospital (Smithers and his colleagues, 1952)

Relative	Total number of deaths		Deaths from cancer other than of the breast				Deaths from cancer of the breast			
	Penrose and his colleagues	Royal Cancer Hospital	Penrose and his colleagues		Royal Cancer Hospital		Penrose and his colleagues		Royal Cancer Hospital	
			Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
Mother	406	556*	51	49·23	61	61 2	25	11·12	29	13·9
Sister	307	460	19	25·23	35	—	23	6·97	16	—
Daugher	30	—	0	0 41	—	—	0	0 12	—	—
Father	420	596*	41	52·32	50	—	0	0·10	2	—
Brother	455	639	29	28 14	24	—	1	0 05	0	—
Son	50	—	2	0 81	—	—	0	0·00	—	—
Total	1,668	2,251	142	156 14	—	—	49	18·36	—	—

* Deaths known   In 12 cases the patients did not know whether their mothers were alive or dead   In 17 cases the patients did not know whether their fathers were alive or dead.

Carcinoma of the uterus and cervix

The high incidence of uterine cancer in the "G" family of Warthin has been mentioned (Table XVI). Fifteen out of 23 cases of female cancers were localized in the uterus (Hauser and Weller, 1936). Waaler (1931) and Wassink (1935)

TABLE XVI

THE CANCER INCIDENCE IN 6 GENERATIONS OF WARTHIN'S "G" FAMILY

(After Hauser and Weller, 1936)

Generation	Number under the age of 25 years	Number over the age of 25 years	Number with cancer
P	—	1	1
1	—	10	6
2	8	54	27
3	30	104	7
4	87	5	—
5	6	—	—
Total	131	174	41 [23 ?]

Penile cancer

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TABLE XVII

DISTRIBUTION OF CANCER INCIDENCE IN FAMILIES OF PATIEN? · WITH
CANCER OF THE UTERUS AND OF CONTROL PERSONS

(*Broebeck*, 1949)

	Patients	Controls
Cancer-free families	20	30
Families with 1 case of cancer ..	22	30
Families with 2 cases of cancer ..	23	20
Families with 3 cases of cancer ..	12	4
Families with 4 cases of cancer ..	8	5
Families with 5 cases of cancer ..	3	0
Families with 6 cases of cancer ..	2	1
Total number of cancer cases ..	163 (18)*	108 (12)

* Numbers in parentheses indicate number of patients with cancer of the uterus

reported that cancer of the uterus was more frequent in relatives of patients than
in the general population. Broebeck (1949) analysed 90 family histories with
endometrial cancer and found that the incidence of cancer at all sites is 50 per cent
higher in relatives of patients with carcinoma of the uterus than in controls (Table
XVII). As male and female relatives of patients showed a significant increase in
the incidence of cancer of all sites, Broebeck concluded that cancer of the uterus
is dependent for its development on a general "cancer gene" common to cancers
of all sites, and also on a genetic factor or factors, which localize the growth in
the uterus. He found no increased familial trend for cancer of the cervix in 200
probands. Murphy (1952) in a survey of 201 probands with cancer of the uterus,
collected from 33 different hospitals, also observed a higher frequency of uterine
cancer in the relatives. The quantitative effect of genetic factors is, however,
small, indicating the interaction of several genes.

Leukaemia

The familial incidence of leukaemia was studied by Videbäck (1947); Busk,
Clemmesen and Nielsen (1948); Anderson (1951); and Ward, Galinsky and
Newton (1952). They found several families in which more than 2 members were
affected. Neel (1953) considered that in view of the relatively high incidence of
leukaemia in the general population (3 per 1,000 total deaths) the occurrence of
3 or 4 cases within a family group can be due to chance alone. When, however,
the frequency of leukaemia is compared in relatives of patients with the disease
and in controls, Videbäck's studies suggest a genetic basis. He found among the
4,041 relatives of 209 leukaemic patients, 319 persons with the disease, while
among the 3,641 relatives of control families there were 218 cases. The difference
is statistically significant for the fathers and sisters of patients (Busk, Clemmesen
and Nielsen, 1948). According to Videbäck the development of leukaemia depends
on a "common non-specific disposition to cancer" which is present in about 20

(1956) found that the estimated mutation rate for the gene of retinoblastoma is four times higher than the actual rate. This is so because many cases of retinoblastoma as shown by the follow-up of children of surviving sporadic cases of this disease, are proved to be due to somatic and not germinal mutation. Koller (1948) described a mild case of xeroderma which suggested a change in the position of the gene responsible for this precancerous condition.

The statistical analysis of the various family pedigrees with a high incidence of cancer, however, definitely shows that the common types of human cancer (for example carcinoma of the breast, uterus stomach), depend on multiple genes which have a cumulative action, but which individually have not a readily discernible phenotypic effect, that is they constitute a polygenic system. As susceptibility to cancer depends on this genetic system it may be expected that too many initially independent, but mutually interacting genes are occasionally brought into effective relationship and are kept together in one family group. This would explain the high incidence observed in certain generations of cancer families. The decline in frequency, or the complete disappearance, of the disease from such families can be attributed to the disruption of the polygenic system due to the segregation of its components. The pedigree of Warthin's "G-family" represents a good example of the "accumulation" and "disruption" of the polygene responsible for the susceptibility to cancer.

According to modern genetical concept, *genes fix only the limits of potential, and the end-product of a gene (that is the character) is the result of many interactions between genotype and environment*. Experiments with mice showed that it is only under certain conditions that the genotype plays the role of a deciding factor in determining whether or not cancer will develop. Several examples are known in which environmental influences have prevented the development of certain tumours in mice to which the individuals were genetically predisposed.

Transplantation experiments in animals indicate that the degree of response of a particular tissue to carcinogenic stimuli, and the time of onset of tumour development, are controlled by the genotype. There is a high probability that this exists in man as shown by the study of cancer in twins, though often it is obscured by environmental influences. The cause of the modifying effect of environment can be explained on evidence established by physiological genetics. The manifestation of genetic characteristics is the result of a series of biochemical reactions, and the longer this biochemical chain from gene to character, the greater is the effect of the environment on the final result. It is now realized that the general basis of susceptibility of cancer in man is a complex phenomenon of polygenic inheritance; variability in expression and in penetrance of character; gene controlled time of incidence; and manifold interaction between modifying genes and environment, the analysis of which demands many entirely new concepts.

Our study of the problem is complicated further by the "uniqueness of man" and by the highly artificial civilization in which he lives. On account of these and experimental procedures are restricted; for example no controlled mating is possible. Many of the conclusions derived from experiments with *Drosophila* and mice, such as the role of natural selection, cannot be interpolated because man is "neither an overgrown fruit fly nor an oversized mouse" (Neel, 1955). New methods are required in the study of pedigrees and new techniques in the statistical treatment of the human data. These are now being employed in the joint investigations

per cent of the population, and its localization to the haemopoietic system is determined by one or more genes.

The evidence seems to indicate that leukaemia is not randomly distributed in human populations but shows a tendency to appear in relatives. Videbáck found a higher incidence of pernicious anaemia among the relatives of leukaemic patients than in controls and a similar observation was made by Lentz (1947). It is not improbable that these diseases affecting the haemopoietic system may have a common predisposition.

Gastric cancer

Familial studies of gastric cancer show a higher incidence of cancer among the relatives of patients with the disease than among the controls. (Wassink, 1935; Denk, 1939; Gardner and Shaffer, 1953; Gardner and Richards, 1953; Macklin, 1953). There are individual family histories in which there is a tendency towards localization of cancer of the gastro-intestinal tract in members of a single family. The high incidence of the same cancer observed in some families suggests a genetically determined predisposition (Gardner and Stephens, 1950; Gardner and Woolf, 1952).

The frequency of gastric carcinoma was determined in the families of 106 patients by Hagy (1954); 66 out of the 1,094 relatives of patients with gastric cancer also had cancer at some site; while in the control series 46 out of 824 had cancer. The frequencies are thus the same (6·0 per cent and 5·6 per cent respectively) if the total cancer incidence is considered. However, the difference between patients and controls becomes significant when the frequency of gastric cancer alone is compared in the two groups; among the relatives of gastric cancer patients, 1·2 per cent (or 1·6) had gastric cancer, while among relatives of control families the incidence was only 0·5 per cent for the same site. Yet the data of Hagy are not sufficient to conclude that there is a genetic component in the development of gastric carcinoma. State, Varco and Wangensteen (1947) observed a higher, but statistically insignificant, incidence of gastric cancer among relatives of 200 patients than in the relatives of control groups. Stephens (1954) reported on 200 families with gastric cancer and found no increased incidence of general cancer death in the families of patients as compared with the general population. On the other hand, the number of deaths due to stomach cancer was significantly higher in his families, and Stephens concluded that an organ specific predisposition is present in the families under observation.

It is noted that pernicious anaemia, a disease which also exhibits definite familial tendencies, frequently occurs together with gastric carcinoma. Washburn and Rozendale (1938) reported 24 gastric lesions and 16 gastric carcinomas in their 906 patients with pernicious anaemia.

Another relationship has been detected between certain blood groups and various carcinomas, including those of the stomach. It is claimed by Aird, Bentall and Fraser-Roberts (1953) on statistical evidence that A blood group is significantly commoner in patients suffering from cancer of the stomach than in the controls in the same hospital. Further investigation suggests that peptic ulcer, a disease believed to have some relationship with gastric cancer, is more frequent in individuals who belong to Group O (Aird and his colleagues, 1954).

organized by geneticists, statisticians and clinicians undertaken simultaneously in several countries. There is no doubt that these will bring together reliable data to give a clearer understanding of the rôle of heredity in human cancer and may lead to a more logical approach towards early diagnosis and prevention.

Genetically determined traits, whether good or bad, are man's heritage. In spite of a fatalistic attitude, however, the present difficulties should be a spur to consider the inexorable but not always uncontrollable effects of the genetic constitution and to try to alter the fate allotted to man by heredity. The study of the genetical aspect of cancer is an urgent task, not only for genetics but also for medicine.
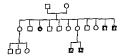
ACKNOWLEDGEMENTS

REFERENCES

Aird, I., Bentall, H. H., and Fraser-Roberts, J. A. (1953). "A Relationship between Cancer of Stomach and the ABO Blood Groups." *Brit. med. J.*, 1, 799.

—— ——Mehigan, J. A., and Fraser-Roberts, J. A. (1954). "Blood Groups in Relation to Peptic Ulceration and Carcinoma of Colon, Rectum, Breast and Bronchus." *Ibid*, 2, 315.

Algire, G. H. (1944). "Growth and Pathology of Melanoma S91 in Mice of Strains DBA, A and C." *J. Nat. Cancer Inst.*, 5, 151.

Altenburg, E. (1933). "The Production of Mutations by Ultraviolet Light." *Science*, 78, 587.

Anderson, R. C. (1951). "Familial Leukaemia." *Amer. J. Dis. Child.*, 81, 313.

Andervont, H. B. (1941). "Note on the Transfer of the Strain C3H Milk Influence through Successive Generations of Strain C Mice." *J. nat. Cancer Inst.*, 2, 307.

—— (1945). "Fate of the C3H Milk Influence in Mice of Strains C and C57 Black." *Ibid*, 5, 383

Arley, N., and Iversen S. (1953). "On the Mechanism of Experimental Carcinogenesis." *Acta path. microbiol. scand.*, 33, 133.

Armitage, P., and Doll, R. (1954). "The Age Distribution of Cancer and a Multistage Theory of Carcinogenesis." *Brit J. Cancer*, 8, 1.

Auerbach, C (1939) "Tests of Carcinogenic Substances in Relation to the Production of·· ·, · · · · · ·· ····· " *p-oc. roy. Soc. Edinb.*, 60, 164.

—— (1' ' ' ' · ' ' · · ' ' ' ' '1, 355.

—— (1 ' ' · · ' ·' · ' '' Cold Spr. Harb. Symp. quant. *Biol.*, 16, 199.

Barrett, M. K., and Deringer, M. K. (1950). "An Induced Adaptation in a Transplantable Tumour in Mice." *J. nat. Cancer Inst.*, 11, 51–59.

Bauer, K. H. (1928). "*Mutationstheorie der Geschwulstenstehung. Übergang von* '' ·· ·····'' · ··· · · ·Gen-Änderung." Berlin; Springer.
'' ·· ·····'' Geschwulstzellen durch Gen-Änderung." Berlin; Springer.
''·· ·' ' · ·· ''· ·pathologie der Stutzgewebe bei Menschen." *Handbuch des Menschen*. (Ed. G. Just.) Vol, 3, p. 105.

Bayreuther, K. (1952). "Die Chromosomenbestand des Ehrlich-Ascites-Tumors der Maus." *Z. Natur.* 7b, 554.

Hereditary basis of other cancers

Retinoblastoma

Studies of family history indicate that the disease is determined by one dominant gene (Weller, 1941). Since it is always fatal, its sporadic appearance is attributed to new mutation, the rate of which has been calculated to be 1.4×10^{-5} (Philip and Sorsby, unpublished work) and 2.3×10^{-5} by Neel and Falls (1951). The incidence of the disease in the population is 5 in 100,000. The isolated appearance of retinoblastoma in the second generation of a pedigree is shown in Fig 71 Instances are also known in which the occurrence of retinoblastoma misses a generation. The penetrance of the disease is almost absolute
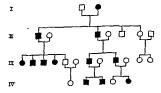

● ■ Retinoblastoma

Fig 71.—Pedigree showing the appearance of retinoblastoma in the second generation, presumably due to mutation *(After Griffith and Sorsby, 1944)*

Neurofibromatosis (vòn Recklinghausen's disease)

Individuals affected are often of subnormal mentality. The disease is due to a dominant gene, which shows a high degree of penetrance and a variable degree of expression (Jones and Hart, 1939).

Multiple exostoses of bone

Another inherited condition is multiple *exostoses* of bone (diaphyseal aclasis). The exostoses are usually present as multiple, more or less symmetrical cartilaginous or osteo-cartilaginous growths in the skeleton. The condition is benign in nature and transmitted in heredity by a dominant gene (Fig. 72) There is a high degree of variability in the expression of the disease within the same family, but pedigree

■ Multiple exostoses

Fig. 72.—Pedigree showing the transmission of multiple exostoses of bone in 4 generations. *(After Bauer and Bode, 1940)*

REFERENCES

Beatty, R. A. (1954). "How Many Chromosomes in Mammalian Somatic Cell Rev. Cytol., 3, 177.

Belling, F. (1927). "The Number of Chromosomes of Cancerous and Other Tumors," J. Amer. med. Ass., 88, 396

Berenblum, I., and Shubik, P. (1949). "An Experimental Study of the Initiation of Carcinogenesis and a Re-examination of the Somatic Cell Mutation Theory of Cancer." Brit. J. Cancer, 3, 109-118

Billingham, R. E., and Medawar, P. B. (1948) "Infective Transformation of Cells" Brit. J. Cancer, 2, 126-131.

Bird, M J. (1952). "Chemical Production of Mutations in Drosophila. Comparison of Techniques," J. Genet., 50, 480

Bittner, J. J (1935). "A Review of Genetic Studies on the Transplantation of Tumors." J. Genet., 31, 471.

—— (1936). "Tumor Incidence in Reciprocal F_1 hybrid Mice and their High Tumor Stocks." Proc. Soc. exp. Biol., 34, 42.

—— (1937). ' Some Possible Effects of Nursing on the Mammary Gland Tumor Incidence in Mice." Amer. J clin. Path., 7, 430

—— (1939). "Relation of Nursing to the Extra-chromosomal Theory of Cancer in Mice." Amer. J. Cancer, 35, 90.

—— (1949). "Factors Associated with the Genesis of Mammary Cancer in Mice." Roscoe B Jackson Memorial Laboratory 20th Commemoration Amer J Med Sci, p. 218

—— (1950). "Genetic Aspect of Cancer Research" J int Coll Surg 20, 544, 228.

Blond, K. (1947). "Cancer and Heredity."

Blum, H. F. (1950). "On the Mechanism of Cancer Induction by Ultraviolet Radiation." J. nat. Cancer Inst., 11, 463

—— (1955). "Ultraviolet Radiation and Cancer." Rad. Biol (Ed. by A. Hollaender and others), 2, 529 New York, MacGraw-Hill.

Borges, P. R. F., and Kvedar, B. J. (1952). "A Mutation Producing Resistance to Several Transplantable Neoplasms in the C57 Black Strain of Mice." Cancer Res 12, 19.

Boveri, T. (1929). The Origin of Malignant Tumours. Baltimore, Williams and Wilkins.

Boycott, A. E. (1933). "Discussion on Experimental Production of Malignant Tumours" Proc. roy. Soc., 113, 291.

Boyland, E. (1954). "Mutagens." Pharmacol. Rev., 6, 345.

Broca, P. (1866). "Traité des Tumeurs." Paris, 1, 149.

Broebeck, O.(1949). Heredity in Cancer Uteri Universitetsforlaget, Aarhus.

Burdette, W. J (1950). "Lethal Mutation Rate in Drosophila Treated with Methyl-cholanthrene." Science, 112, 303

—— (1954). "Effect of Defective Ring Gland on Incidence of Tumours in Drosophila." J. Nat. Cancer Inst., 15, 367.

—— (1955). "The Significance of Mutation in Relation to the Origin of Tumors." Cancer Res., 15, 201

Busk, T., Clemmesen, J., and Nielsen, A. (1948). "Twin Studies and Other Genetical (A Review.) Investigations in the Danish Cancer Registry." Brit. J. Cancer, 2, 156.

Carr, J G. (1947). "Production of Mutations in Mice by 1 : 2 5 . 6 Dibenzanthracene" Brit J. Cancer, 1, 152

—— (1948) "Chemically Induced Mutation." Ibid., 2, 132.

Caspersson, T. (1939). "Uber die Rolle der Desoxyribonukleinsäure bei der Zellteilung." Chromosoma, 1, 147.

Cawley, E. P. (1952). "Genetic Aspects of Malignant Melanoma." Arch. Derm. Siph. N.Y., 65, 440.

(16

analyses indicate a trend towards the multiple character of the exostoses (Stock and Barrington, 1925).

Choroidal sarcoma

The familial incidence of choroidal sarcoma has been reported by Davenport (1927), who followed the disease in three generations and found that 6 members, including a pair of twins, were afflicted out of 14.

Cutaneous melanoma

A family pedigree showing inheritance of cutaneous melanoma has been published recently by Cawley (1952).

Cancer in twins

The study of twins is the best method of ascertaining the respective rôle of heredity and environment in the causation of cancer. Thus, if identical or mono-zygotic twins shows a higher concordance than fraternal or dizygotic twins, a genetical basis may be postulated for the disease. Several family pedigrees in which twins are affected with cancer are known and have been studied by various in-vestigators. A case was reported by Militzer (1935) where both male members of monozygotic twins had identical symptoms and almost simultaneously developed gastric carcinomas which were symmetrically located in the stomach and also had the same histological characteristics. Klotz (1940) published the incidence of carcinoma of the cervix in 17 pairs of twins, 9 of which were identical. He found that in one pair the carcinoma was present in both partners (concordance), while the other 8 showed discordance, that is only one member was affected. In four pairs of identical twins leukaemia has been found in both members (Gorer, 1938). In view of the greater frequency of dizygotic than monozygotic twins in the general population, the concordance shown by the "one-egg" twins can be interpreted as indicating a hereditary basis of leukaemia (Neel, 1953).

Busk, Clemmesen and Nielsen (1948) studied the distribution of various cancers in 183 twins, 50 of which were monozygotic, and only 7 showed concordance which is not a significant increase when compared with the distribution of cancer in dizygotic pairs (Table XVIII). When, however, the sites of cancer are compared in the two sets, Busk and his colleagues found a more pronounced tendency to cancer-formation in the same site among monozygotic than among the dizygotic twins. This observation is similar to that of Macklin (1940) (Table XIX).

TABLE XVIII

CANCER IN TWINS

(Busk, Clemmesen and Nielsen, 1948)

Type of twin	Concordant observed	Concordant expected	Discordant	Total
Monozygotic	7	5	43	50
Dizygotic	7	9 2	126	133

Charles, D. R., and Luce-Clausen, E. M. (1942). "The Kinetics of Papilloma Formation in Benzpyren-treated Mice." *Cancer Res.*, 2, 261.

Cloudman, A. M. (1932). "A Comparative Study of Transplantability of Eight Mammary Gland Tumors Arising in Inbred Mice." *Amer. J. Cancer Res.*, 16, 568.

Cockayne, E. A. (1933). *Inherited Abnormalities of the Skin and Its Appendages.* London; Oxford University Press.

Darlington, C. D. (1944). "Heredity, Development and Infection." *Nature*, 154, 164.

—— (1948). "The Plasmagene Theory of the Origin of Cancer." *Brit. J. Cancer*, 2, 118.

—— and Thomas, P. T. (1941). "Morbid Mitosis and the Activity of Inert Chromosomes in *Sorghum*." *Proc. roy. Soc.*, 130, 127.

Davenport, R. C. (1927). "A Familial History of Choroidal Sarcoma." *Brit. J. Ophthal.*, 11, 443.

Davies, J. N. P. (1955). "Human Implications of Primary Carcinoma of the Liver in Africans." *J. nat. Cancer Inst.*, 15, Suppl. 1637–1644.

Demerec, M., Wallace, B., Witkins, E. M., and Bertani, G. (1949). "The Gene." *Carnegie Institute, Washington, Year Book*, 48, 156.

Denk, W. (1939). "Zur Frage der Erblichkeit des Carcinoms." *Z. Krebsforsch.*, 49, 237.

Dickie, M. M. (1954). "The Use of F_1 Hybrid and Backcross Generations to Reveal New and/or Uncommon Tumor Types." *J. nat. Cancer Inst.*, 15, 791.

Dukes, C. (1930). "The Hereditary Factor in Polyposis Intestini or Multiple Adenomata." *Cancer Rev.*, 5, 241.

—— (1952). "Familial Intestinal Polyposis." *Ann. Eugen.*, 17, 1.

Ephrussi, B. (1951). "Remarks on Cell Heredity." *Genetics in the 20th Century.* (Ed. by L. C. Dunn), p. 241. New York; Macmillan.

Evans, H. M., and Swezy, O. (1928). "The Chromosomes in Man; Sex and Somatic." *Mem. Univ. Calif.*, 9, 1.

Fahmy, O. G., and Bird, M. J. (1953). "Chromosome Breaks among Recessive Lethals Induced by Chemical Mutagens in Drosophila Melangaster." *Heredity*, 6, Suppl. 149.

Farner, J. B., Moore, J. E. S., and Walker, C. E. (1904). "On the Resemblances Between the Cells of Malignant Growths in Man and those of Normal Reproductive Tissues." *Proc. roy. Soc.*, 72, 499.

Fekete, E., and Griffen, A. B. (1954). "Significance of Recent Developments in Nuclear Cytology and Cytogenetics of the Mouse." *J. nat. Cancer Inst.*, 13, 801.

Furth, J. (1954). "Carcinogenesis by Ionizing Radiations." *Rad. Biol.* (Ed. by A. Hollaender and others), 1, 1145 New York; MacGraw-Hill.

Gardner, E. J. (1951). "A Genetic and Clinical Study of Intestinal Polyposis, a Predisposing Factor for Carcinoma of the Colon and Rectum." *Amer. J. Hum. Hered.*, 3, 167.

—— and Stephens, F. E. (1950). "Cancer of the Lower Digestive Tract in One Family Group." *Amer. J. hum. Genet.*, 2 (1) 41.

—— and Woolf, C. M. (1952). "Intestinal Polyposis and Carcinoma Originating from a Mutation in a Family Group." *Cancer*, 5, 695.

—— and Shaffer, J. O. (1953). "The Inheritance of Cancer Involving Different Areas of the Human Gastrointestinal Tract." *J. nat. Cancer Inst.*, 13, 1089.

—— and Richards, R. C (1953). "Multiple Cutaneous and Subcutaneous Lesions Occurring Simultaneously with Hereditary Polyposis and Osteomatosis." *Amer. J. hum. Genet.*, 5 (2) 139.

Goldschmidt, R., and Fischer, A. (1929) "Chromosomenstudien an Carcinomzellen in vitro." *Z. Krebsforsch.*, 30, 281.

Gordon, M. (1951). "The Variable Expressivity of a Pigment Cell Gene from Zero to Melanotic Tumor Induction." *Cancer Res.*, 11, 676.

—— —— (1952) "Selective Matings and Development of Melanomas." *Proceedings 2nd National Cancer Conference*, 2, 1067. New York; American Cancer Society.

HYPOPHYSECTOMY AND ADRENALECTOMY IN THE TREATMENT OF CANCER OF THE BREAST

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THE discovery that tumours of the prostate and breast were often hormone-dependent was and still is exciting. It holds out great promise for future developments in the treatment of patients suffering from metastasizing carcinoma of the breast Surgeons, general practitioners and radiotherapists probably expected too much of 'endocrine treatment' when it was first introduced and most will confess to considerable disappointment in the results that have been obtained so far. Temporary palliation is the best that has been achieved. Miracles of recovery occur from time to time, but these phenomenal successes must not be confused with the general run of results that may be attained. An optimistic outlook, however, prevails in the scientific departments of our cancer centres and further developments may be expected.

THE PROS AND CONS

Endocrine treatment does not claim to do more than alter 'the internal carcinogenic environment' of the body (Cramer and Horning, 1939). If tumour cells are dependent on œstrogens, androgens can *sometimes* neutralize the stimulus and create circumstances temporarily unfavourable to the continued growth of the tumour. The simple administration of methyl testosterone by mouth (25 to 50 mg. or more a day), or testosterone propionate by injection (100 mg. intramuscularly at least twice a week), is thus the first step in hormone treatment (Galton, 1950). If successful, it postpones the more drastic surgical methods and indicates that surgery will in its turn be likely to result in further benefit. The administration of androgens is not only a therapeutic measure but is a clinical test, the only one which is a pointer as to how the patient will react. On the other hand, failure with testosterone does not indicate that surgery should not be considered.

When the tumour ceases to respond to androgens, or if it fails to regress under this treatment, one of two operations may be done. The first is the removal of the source of œstrogens, namely the ovaries and the adrenals. The second is the removal of the stimulus to the production of œstrogens by the destruction of the pituitary gland which produces the gonadotrophic and adrenocorticotrophic hormones.

Androgen treatment and endocrine surgery are usually indicated in

patients who are not old or who are young for their age. The upper limit of age for this therapy is about 60, but this is not necessarily so, especially for patients with severe bone pain. For the senile, or those who appear old for their years, the reverse of androgen therapy, namely the administration of œstrogens, may be worth a trial, but never following hypophysectomy or adrenalectomy.

The following case report gives an example of the sequence of events and the reasons which influence the choice of treatment.

In October 1954, a woman of 40 became completely paralysed from the level of the nipples downwards, due to collapse of the 4th dorsal vertebra from metastases from a carcinoma of the breast. Many courses of x-ray treatment had been given and both ovaries had been removed, with little effect on the progress of the

that by herself she took her two children on a caravan holiday. In October of that year, however, pain in the back returned and the legs suddenly weakened. Without delay both adrenals were removed at one operation. The patient soon recovered. In May 1957, she suffered a pathological fracture of the shaft of her right femur while turning over in bed. This has been fixed with a Küntscher nail and she can walk again.

Bony metastases, however, are growing again and hypophysectomy has been considered and offered; but the patient's morale is broken and it seems almost cruel to try to restore her yet again to an active life so that her hopes may be dashed for a third time. Hypophysectomy has therefore been considered unwise in this

would be mistaken.

This case history illustrates in practice the applications of the theories which have been discussed by Professor George Lumb (p. 241). Should it be the adrenals and ovaries or the pituitary that is removed? What should be the sequence of events? How late should such drastic surgery be deferred, or should it be done early in the terminal phase of the disease? These and other questions must be carefully weighed.

BILATERAL OÖPHORECTOMY AND ADRENALECTOMY

This operation, successfully introduced by Huggins, is usually done in two stages. The first consists of the removal of the ovaries through a midline subumbilical incision, and the left adrenal through an incision in the left flank; the second, done seven to fourteen days later, involves the removal of the right adrenal through a similar incision in the right flank. The adrenalectomy incisions are placed along the lines of the 12th ribs and extended a short distance forwards, the patient being fixed on the operating table on her side with the table broken to open out the wound. This approach gives the best exposure and makes for an easy and complete operation. Since the purpose of adrenalectomy is to remove all the adrenal tissue, including outlying rests, it is necessary to dissect the peri-adrenal fat away from the surrounding structures. For example, on the right side it is advisable to wipe this fat and the upper perinephric fat off the inferior vena cava and the

Gorer, A. (1938). "The Genetic Interpretation of Studies on Cancer in Twins." *Ann. Eugen. Lond.*, 8, 219.

—— (1948). "Significance of Studies with Transplanted Tumours." *Brit. J. Cancer*, 2, 103.

—— (1953). "Cancer" In *Clinical Genetics*, p 558. London, Butterworth.

Graffi, A. (1940). "Intercelluräre Benzpyrenspeicherung in lebenden Normal- und Tumorzellen. *Z. Krebsforsch.*, 50, 196

—— (1953). "Untersuchungen über der Mechanism der Carcinogenese und der Wirkungsweise carcinogener Reize." *Abh. Dsch. Akad Wiss, B.cl.*, 1, 1

Griffith, A. D., and Sorsby, A. (1944). "Genetics of Retinoblastoma." *Brit J. Ophthal.*, 28, 279.

Gross, L. (1954). "Transmissible Mouse Leukaemia, Biological Properties of the Mouse Leukaemia Agent." *Leukaemia Research*. Ciba Symposium. (Ed. by E. W. Wolstenholme and M. P. Cameron) London; Churchill

Grüneberg, H. (1952). *The Genetics of the Mouse*. The Hague, Martinus Nijhoff.

Habs, H. (1939). "Krebs und Vererbung." *Z f. klin Med*, 135, 676

Haddow, A. (1944). "Transformation of Cells and Viruses." *Nature*, 154, 194 199.

Hagy, G. W. (1954). "A Familial Study of Gastric Carcinoma." *Amer. J. hum Genet*, 6, 434.

Haldane, J. B. S. (1936). "A Search for Partial Sex-linkage in Man." *Ann. Eugen. Lond.*, 7, 28.

Harnly, M. H. (1955). "The Tu-e-gene Action and Tumor Development." *Trans New York Acad. Sci.*, 17, 309.

Hansemann, D. von. (1890). "Über asymmetrische Zellteilung in Epithelkrebsen und deren biologische Bedeutung." *Virchow's Arch*, 119, 299.

—— (1891). "Über Kernteilungsfiguren in bösartigen Geschwülsten." *Biol Zbl* 24 189.

—— (1892). "Über die Anaplasia der Geschwulstzellen und die asymmetrische Mitose." *Virchow's Arch.*, 129, 436.

—— (1906). "Über die Funktion der Geschwülstzellen." *Z. Krebsforsch.*, 4, 565.

Hauschka, T. S. (1952). "Some Influences on the Transplantation and Growth of Tumors." (Discussion) *Proceedings 2nd National Cancer Conference*. 2. 1044 New York; American Cancer Society.

—— (1953). "Cell Population Studies on Mouse Ascites Tumors." *Trans. N.Y. Acad. Sci.*, S.II., 16, 64.

—— and Levan, A. (1951). "Characterization of Five Ascites Tumors with Respect to Chromosome Ploidy." *Anat. Rec.*, 3, 467.

—— (1953). "Inverse Relationship between Chromosome Ploidy and Host-Specificity of Sixteen Transplantable Tumors." *Exper. Cell. Res.*, 4, 457.

Hauser, I. J., and Weller, C. V. (1936). "Further Report on the Cancer Family of Warthin." *Amer. J. Cancer*, 27, 434.

Heiberg, K. A., and Kemp, T. (1929). "Über die Zahl der Chromosomen in Carcinomzellen beim Menschen." *Virchow's Arch.*, 158, 693.

l'Heritier, P. (1951). "The CO_2 Sensitivity Problem in Drosophila." *Cold Spr. Harb. Symp. quant. Biol.*, 16, 99.

Heston, W. E. (1940). "Lung Tumors and Heredity. I. The Susceptibility of Four Inbred Strains of Mice and their Hybrids to Pulmonary Tumors produced by Subcutaneous Injection." *J. nat. Cancer Inst.*, 1, 105.

—— (1941). "Relationship between Susceptibility to Induced Pulmonary Tumors and Certain Known Gene in Mice." *Ibid.*, 2, 127.

—— (1942a). "Genetic Analysis of Susceptibility to Induced Pulmonary Tumors in Mice." *Ibid.*, 3, 69.

transition which occurs also in the $\diagdown$C=O group, i e the $n \to \pi$ transition of a non-bonding or lone-pair electron, as on the N or O atom excited to an unfilled π-electron energy level (see PLATT [5]) For pyridine the transition is evident only as a shoulder on the red slope of the $\pi \to \pi$ system so that the ε_{max} given in Table 1 is the more intense one for this $\pi \to \pi$ system In the prevailing spectrum the $n \to \pi$ transition is found as a separate band and we notice that as for C=O, it is weakly allowed. As long ago as 1935 MULLIKEN [20] drew attention to this type of transition in formaldehyde and a useful summary of their properties has been given recently by KASHA [21]. This article [21] also includes a brief discussion of
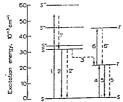
Fig 1. Known radiative (full lines) and radiationless processes (broken lines) in naphthalene

1. Excitation to $S', \ldots S''''$;
1'. rapid internal conversion from $S''''\ldots$ to S'
2. Fluorescence; 2' fluorescence quenching
3.
4.
5.
6.   n to T.

the properties of the lowest triplet states of molecules, i.e. those excited states in which electron pair uncoupling occurs, transitions to these states being less probable and therefore weaker than the usual singlet-singlet transitions described above. These triplet states will not be discussed in detail here, but it should be remembered that the lowest triplet state may be of considerable importance in photochemical or other energy transfer processes, particularly because of its long lifetime. Low-temperature phosphorescence is the simplest way of detecting the lowest triplet state in most molecules, since under these conditions the viscosity of the rigid glass solvents used reduces the radiationless collisional and interaction processes (or quenching) which occur normally in fluid systems. An interesting and speculative discussion of the possible importance of $n \to \pi$ singlet and $\pi \to \pi$ triplet states in energy transfer processes in chlorophyll is given in the article by KASHA [21].

Fig. 1 summarizes in diagrammatic form the variety of possible energy changes both radiative and radiationless, which can occur in a molecule after the initial absorption of electronic energy. It refers to a particular example, naphthalene (CRAIG and Ross [22]). $S' \ldots S''''$ denote singlet states, i.e. states containing

paired electrons of opposite spin, and T, T' are triplet states, containing electrons of parallel spin, the electron-pair uncoupling process occurring in inter-system crossing (process 3) between singlet and triplet states. Transitions shown on the left hand side of the figure, i.e. between singlet states only, will be considered further in this paper, although it should be remembered that the triplet states of molecules have been the subject of considerable experimental investigation in recent years. since LEWIS and his associates redirected attention to this important topic [23, 24].

Some effects of intramolecular environment on absorption

A few common intramolecular changes which affect absorption spectra in the
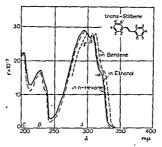
Fig 2. Absorption spectrum of *trans*-stilbene in *n*-hexane, in ethanol (95%) and in benzene.

u.v. and visible region may be illustrated simply by reference to the molecule *trans*-stilbene.

Stilbene's 'finger-print bands' (Fig. 2 and reference [17]), are simple in outline although vibrational structure appears in the long-wavelength band even at room temperatures. This band varies somewhat in position and extinction with solvent, as is expected, and will be discussed further below. In Fig. 3 (EtOH solution) the usual effects of a fairly strong auxochromic substituent, the —NH_2 group, are evident in the shift of absorption to the red, the changes in the band-envelope and the reduction of fine structure when this group is substituted at the 4-position in stilbene, in direct conjugation with the length of the molecule. COULSON and JACOBS [25] have calculated the change in π-electron charge distribution for ground and excited states in this molecule, 4-aminostilbene. Their results are shown diagrammatically in Fig. 3, where in the formulae, the radii of the black circles are drawn proportional to the calculated excess π-electron charge (above 1·0) on each carbon atom. It is seen that the tendency of the —NH_2 group to donate electronic charge to the conjugated hydrocarbon system is facilitated in the 'loosened-up' condition of the excited state. More charge reaches the 4'-position than in the

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Koller, P. C. (1953a). "Dicentric Chromosomes in a Rat Tumour Induced by an Aromatic Nitrogen Mustard." *Heredity*, 6 Suppl., 181.

—— (1953b). "Experimental Methods. 1 Cytogenetics." In *Clinical Genetics*. (Ed. by A. Sorsby). London: Butterworth.

—— (1953c). "Practical Applications of the Squash Method in Cytology." *Lab. Practice*, 2, 473.

—— (1956). "Cytological Variability in Human Carcinomatosis." *Trans. N.Y. Acad. Sci.*, 63, 793.

Kostoff, D. (1930). "Tumours and Other Malformations on Certain Nicotiana Hybrids." *Zbl. Bakt.*, 81, 244.

Latarjet, R., Buu-Hoi, N. P., and Elias, C. A. (1950). "Induction d'une mutation spécifique chez une bacterie par des cancerigenes hydrosolubles." *Bull. Soc. Chim. Napoli*, 22, 78.

Law, L. W. (1954). "Genetic Studies in Experimental Cancer." *Advances in Cancer Research*. (Ed. by J. P. Greenstein and A. Haddow) 3, 281. New York: Academic Press.

—— and Miller, J. H. (1950). "The Effect of Thymectomy on the Incidence, Latent Period and Type of Leukaemia in the High Leukaemia Strains of Mice." *J. nat. Cancer Res.*, 10, 230.

—— Dunn, Th. B., and Boyle, P. J. (1955.) "Neoplasms in the C3H Strain and in F1 Hybrid Mice of Two Crosses following introduction of Extracts and Filtrates of Leukaemic Tissues.", *J. nat. Cancer Inst.*, 16, 495.

Lentz, O. (1947). "Krebs und Vererbung." *Arb. Staatl Inst exp Ther.*, Heft 42, 45.

Levan, A., and Hauschka, T. S. (1952). "Chromosome Numbers of Three Mouse Ascites Tumors." *Hereditas*, 38, 251.

Levine, M. (1930). "The Chromosome Number in Cancer Tissue of Man, of Birds, of Bird, and in Crown Gall Tissue in Plants." *Amer. J. Cancer*, 14, 400.

—— (1931). "Studies in Cytology of Cancer. Crown Gall Disease. Historical Document." *Amer. J. Cancer*, 15, 144; 788; 1410.

Lockhart-Mummery, J. P. (1934). *The Origin of Cancer*. London: Churchill

—— (1934). *Diseases of the Rectum and Colon*. Baltimore: Wood

Loeb, L. (1902). "Further Investigations in Transplantation of Tumours." *J. med. Res.*, 3, 44.

Little, C. C. (1914). "A Possible Mendelian Explanation for the Type of Inheritance apparently Non-Mendelian in Nature." *Science*, 40, 904

—— (1939). "Hybridization and Tumor Formation in Mice." *Proc. nat. Acad. Sci.* 25, 452.

Lucké, B. (1934) "A Neoplastic Disease of the Kidney of the Frog (*Rana pipiens*)." *Amer. J. Cancer*, 20, 352.

Ludford, R. J. (1925). "The General and Experimental Cytology of Cancer." *J. R. Micr. Soc.*, 45, 249.

—— (1930a). "Chromosome Formation without Spindle Development in Cancer Cells and its Significance." 9th Scientific Report of International Cancer Research Foundation, 100.

Ludford, R. S (1930b) "The Somatic Cell Mutation Theory of Cancer." 9th Scientific Report of International Cancer Research, 48, 121.

MacDowell, E. C. (1934). "Genetic Aspects of Mouse Leukaemia." *Amer. J. Cancer*, 26, 85.

—— and Richter, M. N (1935) "Mouse Leukaemia. IX The Role of Heredity in Spontaneous Cases." *Arch. Path.*, (Lab. Med.) 20, 709.

—— Potter, J. S., and Taylor, M. J (1945) "Mouse Leukaemia. XII The Role of Genes in Spontaneous Cases." *Cancer Res.*, 5, 65.

ground state and the charge-distribution about the ethylenic double bond changes most of all. If, as in aqueous acid solution, the extra charge on the amino-group is satisfied by means of a proton, the spectrum reverts to that of stilbene (Fig. 3); and the spectroscopic method has been used [26, 27] to determine the dissociation constants of a number of basic stilbene derivatives. In these compounds it was of particular interest to know whether dissociation could be related in any way to biological properties, for some of the bases possess carcinogenic and tumour-inhibitory activities [18]. Changes of dissociation constant on substitution were
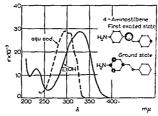
Fig. 3. Absorption spectrum of 4-aminostilbene in ethanol (95%) and in aqueous acid

found to be small, as was expected. Changes in molecular planarity on substitution proved of greater interest, however, for it was found [18] that those members of the series which were shown spectroscopically to be seriously buckled were not

Fig. 4. Plane diagram of 4-dimethylamino-2':6'-dimethylstilbene (a large circle represents the van der Waal's zone of a methyl group and a small circle that of a hydrogen atom).

biologically active. Such an example is 2':6'-dimethyl-4-dimethylaminostilbene, of which a planar diagram is given in Fig. 4. In these compounds, the buckling of the molecule may be determined from the behaviour of the longest-wavelength band. Reduction in the wavelength and in the ϵ_{max} of this band might be expected in clear cases of buckling [18, 19], and, in addition, MULLIKEN [28] has shown that for an allowed (NV) transition such as this [29, 30], the intensity of the band, i.e. its f-value (which is proportional to the area under the band, $f = 4.315 \times 10^{-9} \int \epsilon \, d\tilde{\nu}$) should be very sensitive to changes in bond-angles, even when the calculated

—— and Taylor, M. J. (1948). "Mouse Leukaemia. XIII. A Maternal Influence that lowers the Incidence of Spontaneous Cases." *Proc. Soc. exp. Biol., N.Y.,* 68, 571.

Macklin, M. T. (1940). "An Analysis of Tumors in Monozygous and Dizygous Twins." *J. Heredity,* 31, 277.

—— (1952). "The Genetic Basis of Human Mammary Cancer." *Proceedings, 2nd National Cancer Conference.* Vol. 2, 1074–1084. New York: American Cancer Society.

—— (1953). "Inheritance of Cancer in Man." *Eugen. News,* 38, 112.

—— (1954). "Methods of Selection of Probands and Controls." *Amer. J. hum. Genet.,* 6 (1), 86.

McClintock, B. (1941). "The Stability of Broken Ends of Chromosome in *Zea mays.*" *Genetics,* 26, 234.

Makino, S. (1952). "Presence of the Strain Cells in Ascites Sarsomas of Rats and their Chromosome Constitutions." *Genetics,* 37, 603.

—— and Kano, K. (1953). "Cytological Studies of Tumors. IX. Characteristic Chromosome Individuality in Tumor Strain-Cells in Ascites Tumors." *J. Nat. Cancer Inst.,* 13, 1213.

—— and Kanehisa, T. (1954). "Some Aspects of the Melanotic Tumor occurring in *Drosophila virilis.*" *Gann.,* 45, 17.

Manna, G. K. (1954). "Chromosome Number of Human Endometrium." *Nature,* 173, 271.

—— (1955). "Chromosome Number of Human Cancerous Cervix Uteri." *Naturwissenschaften,* 42, 253.

Mather, K. (1943). "Polygenic Inheritance and Natural Selection." *Biol. Rev.,* 18, 32.

Militzer, R. E. (1935). "Carcinoma of the Stomach in Identical Twins." *Amer. J. Cancer.,* 25, 544.

Mitchell, J. S., and Haybittle, J. L. (1955). "Carcinoma of the Skin appearing 40 years after a single diagnostic Roentgen exposure." *Acta Radiol. scand.,* 44, 345.

Muller, H. J. (1927). "The Problem of Genic Modification." *Science,* 66, 84.

—— (1951). "Radiation Damage to Genetic Material." *Science in Progress,* 7, 93.

—— (1954). "The Nature of the Genetics Effects Produced by Radiation." *Rad. Biol.,* 1, 351.

Murphy, D. P. (1952). *Heredity in Uterine Cancer.* Cambridge, Mass.; Harvard Univ. Press.

Murray, J. A. (1933). "Discussion on Experimental Production of Malignant Tumours." *Proc. roy. Soc.,* 113, 268.

Nachtscheim, H. (1956). "Mutationsrate und mutagene Faktoren, Selektionsdrück und Kontraselektion in ihrer Wirkung auf Freqwenz und Verbreitung krankhafter Gene in menschlichen Populationen." *Proc. World Population Conf.* (in press).

Neel, J. V. (1953). "Haemopoietic System." In *Clinical Genetics* (Ed. by A. Sorsby), p. 446. London; Butterworth.

—— (1954). "Problems in the Estimation of the Frequency of Uncommon Inherited Traits." *Amer. J. hum. Genet.,* 6, 51.

—— (1955). "On some Pitfalls in Developing in Adequate Genetic Hypothesis." *Ibid.,* 7, 1.

—— and Falls, H. F. (1951). "The Rate of Mutation of the Gene Responsible for Retinoblastoma in Man." *Science,* 114, 419.

Nordling, C. O. (1953). "A New Theory on the Cancer-Inducing Mechanism." *Brit. J. Cancer,* 7, 68.

Oftedal, P. (1953). "The Histogenesis of a New Tumour in *Drosophila melanogaster* and a comparison with Tumours of Five Other Stocks." *Z. indukt. Abstamm.-u. VererbLehre,* 85, 408.

frequency of the transition is approximately independent of the shape of the molecule. Thus, one could imagine that as a result of buckling such a conjugated system the molecule in the ground state would approximate less frequently to the planar configuration which is demanded for ease of excitation to the higher-energy states. This would result in a lower transition probability and lower intensity. In order to test this prediction, f-values were compared by measuring the area under this long-wavelength band, on a frequency scale, after separating it graphically from its neighbour as consistently as possible. Examples are shown in Fig. 5
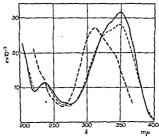
Fig 5. Absorption spectra (95% EtOH) of 4-dimethylaminostilbene(—), 4-dimethylamino-2'-methylstilbene (-----) and 4 dimethylamino-2':4':6'-trimethylstilbene (·········).

of the spectroscopic results of buckling, which is obvious in the 2': 4': 6'-trimethyl-derivative from the wavelength shift alone, but less obvious in the 2'-methyl compound, where no wavelength shift occurs. An example of the band-separation

Table 2. Effect of Methyl Substitution on f-Value of Long-wavelength Band in Stilbene and Derivatives

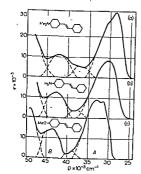| Group	Δf_{aver}
(i) Non-hindering Methyl (4- or 4'-)	+0 050
(ii) Non-hindering Methyl (3- or 3'-)	+0 005
(iii) Single hindering Methyl	−0·040
(iv) Second hindering Methyl (same ring as (iii))	−0·100
(v) Second hindering Methyl (different ring from (iii))	−0·075
(vi) Fourth hindering Methyl	−0 140

carried out is shown in Fig. 6 (a), where the low-frequency band has been cut off from two higher-frequency bands in 4-dimethylaminostilbene (4DMAS). When methyl derivatives of stilbene and of 4DMAS are considered, a range of f-values is

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Oster, I. I. (1954). "Factors Bearing on the Non-Malignancy of Tumors in *Drosophila*." *Cancer Res.*, 14, 478.

Painter, T. S. (1923). "Studies in Mammalian Spermatogenesis. II. The Spermatogenesis of Man." *J. exp. Zool.*, 37, 291.

Penrose, L. S., Mackenzie, H. J., and Karn, M. N. (1948) "A Genetical Study of Human Mammary Cancer." *Brit. J. Cancer*, 2, 168

Petersen O. (1954). "Radiation Cancer." *Acta Radiol scand.*, 42, 221.

Poll, H. (1920). "Zwischenzellengeschwulste des Hodens bei Vogel-mischlingen." *Beitr. path. Anat.*, 67, 40.

Pybus, F. C., and Miller, E. W. (1938). "A Sex Difference in the Incidence of Bone Tumors in Mice." *Amer. J. Cancer*, 34, 248.

Sacharov, W. W. (1938). "On the Specificity of the Action of the Factors of Mutation." *Biol. Zh.*, 7, 595.

Satina, S., Rappaport, J., and Blakeslee, A. F. (1951) Ovular Tumors connected with Incompatible Crosses in *Datura*." *Amer. J. Botany* 37, 576

Schintz, H. R., Cocchi, U., and Neuhaus, J. (1948) "Die Vererbung des Krebses bei Menschen." *Arch. Klaus.-Stift. VererbForsch.*, 23, 1

Shalin, M. P., Keen, P., and Murray, J. F. (1953). "Skin Cancer in the South African Bantu." *Brit. J. Cancer*, 7, 45.

Shatoury, El. H. H. (1955). "A Genetically Controlled Malignant Tumour in *Drosophila* *Arch. EntwMech. Org.*, 147, 496.

Shimkin, M. B. (1955). "Pulmonary Tumors in Experimental Animals." *Advances in Cancer Research*, 3, 223. (Ed. by J. P. Greenstein and A. Haddow.) New York. Academic Press.

—— and McClelland, J. N. (1949). "Induced Pulmonary Tumors in Mice. IV Analysis of Dose Response data with Methylcholanthrene." *J nat. Cancer Inst.*, 10, 597

Shope, E. R. (1933). "Infectious papillomatosis of Rabbits" *J. exp. Med.*, 58, 607.

Smithers, D. W. (1948). "Family Histories of 459 Patients with Cancer of the Breast." *Brit. J. Cancer*, 2, 163.

—— Rigby-Jones, P., Galton, D. A. G., and Payne, P. M. (1952). "Cancer of the Breast." *Brit. J. Radiol.*, Suppl., 4, 1.

Snell, G. D. (1948). "Methods for the Study of Histocompatibility Genes." *J. Genet.*, 49, 87.

—— (1953). "Transplantable Tumors" In *Physiopathology of Cancer*. (Ed. F. Homburger and W. H. Fishman.) London; Cassell.

—— (1954). "The Enhancing Effect (or actively Acquired Tolerance) and the Histocompatibility—2 Locus in the Mouse." *J. nat. Cancer Inst.*, 15, 665.

Sonneborn, T. M. (1951). "The Role of Genes in Cytoplasmic Inheritance." In *Genetics in the 20th Century*. (Ed. L. C. Dunn) New York; Macmillan.

Spiegelman, S. (1951). "The Particulate Transmission of Enzyme forming Capacity in Yeast." *Cold Spr. Harb. Symp. quant. Biol.*, 16, 87.

State, D., Varco, R. L., and Wangensteen, O. H. (1947). "An Attempt to Identify Likely Precursors of Gastric Cancer." *J. nat. Cancer Inst.*, 7, 379.

Steiner, P. E. (1954). *Cancer, Race and Geography*. Baltimore; Williams and Wilkins.

Stephens, F. E. (1954). "Sampling Techniques Available in Human Genetics." *Amer. J. hum. Genet.*, 6 (1), 60.

Stewart, H. L., and Andervont, H. B. (1938). "Pathologic Observations on Adenomatous Lesion of Stomach in Mice of Strain I." *Arch. Path.*, 26, 1009.

Stock, P. (1953). "A Study of the Age Curve for Cancer of the Stomach in connection with a Theory of the Cancer Producing Mechanism." *Brit. J. Cancer*, 7, 407.

—— and Barrington, A. (1925). "Hereditary Disorders of Bone Development." *Treas. hum. Inherit.*, 3, 1.

found for this band (19). varying from 0·475 for the seriously buckled hexamethyl-stilbene to about 0 925 for the planar 3': 4'-dimethyl-4DMAS. The results can be summarized in Table 2 in terms of the difference (Δf) between f-values for a pair of compounds, one with and the other without a methyl group at a particular position e.g. at a non-hindering position such as 4'- or 3'- or at the hindering 2'-position. This Δf-value is very constant when referred to the same group (such as the methyl group at the 4-position in the pairs. 4-methylstilbene—stilbene ($\Delta f = +0 045$); and 4'-methyl-4DMAS—4DMAS ($\Delta f = +0 050$). etc.). While there occurs a small
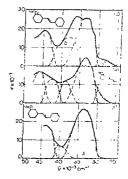
Fig. 6. Effects of substituents on the absorption spectrum of *trans*-stilbene (in 95% EtOH)
(a) 4-NMe₂-, (b) 4-NH₂-, (c) 4-MeO-, (d) 3 NMe₂-, (e) 3 NH₂-, (f) 3-MeO

positive Δf for methyl-substitution at the 4- or 4'-position and a negligible change at the 3- or 3'-position. a hindering methyl group produces a small reduction in intensity. as predicted by MULLIKEN, i.e. a negative Δf, the magnitude of the reduction depending on the position of the hindering substituent; e.g. 2-, 6-. 2'- and 6'-positions can be considered. It seems, therefore, that such investigations of changes in f-values for the long-wavelength band in these compounds is of use to estimate steric hindrance. It may also reveal other effects of substitution. Thus it is found [19] that the f-changes quoted above are additive and independent for the different positions of substitution, suggesting that the effects of the methyl substituent in this molecule are localised and that these groups are perturbing the molecule to a very small extent. PLATT [31] has shown theoretically that this is to be expected and that the Δf-values are of the calculated order of magnitude.

Small changes in the shape of an apparently simple band-envelope with chang-ing substituents may be used sometimes to detect hidden transitions. Such complexity has been revealed in the stilbene spectrum by comparing the effects of increasingly perturbing substituents at any one position in the molecule [32]

Strong, L. C. (1926). "A Genetic Study of the Growth of a Transplantable Tumor (Adenocarcinoma dBrB)." *J. exp. Zool.*, 45, 231.

—— (1940). "A Genetic Analysis of the Induction of Tumors by Methylcholanthrene with a Note on the Origin of the NH Strain of Mice." *Amer. J. Cancer*, 39, 347.

—— (1945a). "Genetic Analysis of the Induction of Tumors by Methylcholanthrene. VIII. Two Mutations Arising in Mice Following Injection of Methylcholanthrene." *Arch. Path.*, 39, 232.

—— (1945b). "Genetic Analysis of the Induction of Tumors by Methylcholanthrene. XI. Germinal Mutations and Other Sudden Biological Changes Following Subcutaneous Injection of Methylcholanthrene." *Proc. Nat. Acad. Sci., Wash.*, 31, 290.

—— (1945c). "Genetic Analysis of the Induction of Tumors by Methylcholanthrene. XII. The Effects of Selection toward Resistance." *Yale J. Biol. Med.*, 18, 145.

—— (1946) "The Induction of Germinal Mutations by Chemical Means (Methylcholanthrene)." *Genetics*, 32, 108.

—— (1947). "The Induction of Germinal Mutations by Chemical Means." *Amer. Nat.*, 81, 50.

—— (1949a). "The Induction of Germinal Mutations by Chemical Means." *Proceedings, 8th International Congress Genetics.* Suppl. *Hereditas*, p. 486.

—— (1949b). "The Induction of Mutations by a Carcinogen." *Brit. J. Cancer*, 3, 97.

—— (1952). "Twelve Mutations in One Descent of Mice Injected with Methylcholanthrene." *Cancer Res.*, 12, 300.

Tatum, E. L. (1947). "Chemically Induced Mutations and Their Bearing on Carcinogenesis." *Ann. N.Y. Acad. Sci.*, 49, 87.

Therman, E., and Timonen. S. (1951). "Inconstancy of the Human Somatic Chromosome Complement." *Hereditas*, 37, 266.

—— —— (1954). "The Prophase Index and the Occurrence of Multipolar Divisions in Human Cancer Cells." *Ibid.*, 40, 313.

Timoféeff-Ressovsky, N. W. (1937). *Mutationsforschung in der Vererbungslehre.* Dresden, Steinkopff.

Timonen, S. (1950). "Mitosis in Normal Endometriums and Genital Cancer." *Acta Obstet. Gynec scand*, 31, Suppl. 2, I.

—— and Therman, E. (1950a) "The Changes in the Mitotic Mechanism of Human Cancer Cells." *Cancer Res*, 10, 431.

—— —— (1950b). "Variation of the Somatic Chromosome Number in Man." *Nature*, 166, 995

Tjio, J. H., and Levan, A. (1956) "The Chromosome Number of Man." *Hereditas*, 42, 1.

Toolan, H. W. (1955). "Subcutaneous Growth of Normal and Malignant Human Tissues in Heterologous Host " *Trans N.Y. Acad Sci.*, 17, 589.

Verschuer, O. von., and Kober, E (1940). "Die Frage der erblichen Disposition zum Krebs. *Z. Krebsforsch* , 50, 5.

Videback, A. (1947) *Heredity in Human Leukaemia and its Relation to Cancer.* Copenhagen; Nyt. Nordisk Forlag.

Waaler, G. H. H. (1931) "Über die Erblichkeit des Krebs." In *Norske Videnskapakademi,* 2, 75. Oslo; Mat. Naturv. Kl.

Wagner, R P., and Mitchell, H. K. (1955). *Genetics and Metabolism.* (Ed. John Wiley.) London, Chapman and Hall

Ward, J. E., Galinsky, I., and Newton B. L. (1952) "Familial Leukaemia. A report of Three Cases of Leukaemia and One Leukaemoid Reaction in One Family." *Amer. J. hum. Genet* , 4, 90

Warthin, A S. (1913). "Heredity with Reference to Carcinoma." *Arch. int. Med Chir.* 12, 546.

—— (1925). "The Further Study of a Cancer Family." *J. Cancer Res.*, 9, 279.

Somewhat similar effects can be observed in the spectrum of diphenyl (WENZEL [33]).

Fig. 6 (c, b, and a) shows the changes in the near u.v. spectrum as substituents causing increasing perturbation (MeO, NH_2, NMe_2) are placed at the 4-position in stilbene. Increase in band-area, i.e. in intensity, and shift to lower frequencies occur from MeO to NMe_2 for the low frequency band, and the rise in the minimum between bands A and B may indicate, it is thought, an increased intensity for a postulated band in this region. Fig. 6 (f, e, d) shows the effect of similar substituents at the 3-position. Here the low-frequency band (A) changes less in intensity and apparently not at all in frequency, so that it is in the same position as in stilbene; but on either side of it appears a new band, most clearly seen in 3-dimethylamino-stilbene (Fig. 6d). The effects in 2-substituted derivatives are similar, but more complex [32]. These changes might be expected if it is assumed with PLATT [5] that this band in stilbene corresponds to a transition polarized longitudinally, i.e. along the long axis of the molecule, so that on applying a perturbation longitudinally, at the 4-position, the symmetry is maintained about this axis. Perturbation at the 3-position, however, would destroy the geometrical symmetry of the molecule and the π-electron orbitals and nodes would be distorted. In resonance terminology, a perturbing 4-substituent can conjugate with the molecule as a whole, but a similar 3-substituent conjugates only with one phenyl group so that a new structure such as

may be expected to make an important contribution to the resonance hybrid. Such 3-substitution should be evident through its effect on any transversely polarized transitions which occur; and it is suggested [32] that such transitions are hidden in the band-envelope on either side of the main longitudinal stilbene transition (A), and are enhanced (by stealing intensity from band A) by 3-substituted auxochromes giving, e.g. bands a and b as shown in Fig. 6(d). (PLATT [5] had already suggested the presence of one transition hidden in the low-frequency slope of this stilbene band.) Measurements with polarized u.v. radiation would test this analysis. However, recent work by LONGUET-HIGGINS and MURRELL [34, 35] provides a possible alternative explanation for these transitions. These authors have shown theoretically that new transitions may arise when a mesomeric substituent interacts with a hydrocarbon. These are due to the electron transfer states which result when an electron is transferred from the substituent to the hydrocarbon, as in the case of an electron donor such as the NH_2 group. The 2350 Å band in the aniline spectrum is attributed to excitation to such an electron transfer state. The choice between these alternative explanations for the stilbene bands cannot be made yet, but the experimental observations have been applied to check the identity of stilbene derivatives having auxochromes at both 3- and 4-positions. The spectrum of one of these, synthesized for its biological interest (BERGEL [36]), is shown in Fig. 7, and in dilute alkaline solution (or in ethanol, for the compound, 3-methoxy-4-aminostilbene, was used as the readily dissociable

Washburn, R. N., and Rozendale. H. M. (1938). "Gastric Lesions, associated with Pernicious Anaemia." *Ann. intern Med.,* 11, 2172.

Wassinck, W. F. (1935). "Cancer at Heredite" *Genetica,* 17, 103.

Weller, C. V. (1941). "The Inheritance of Retinoblastoma and its Relationship to Practical Eugenics." *Cancer Res ,* 1, 517.

Whitman, R. C. (1919). "Somatic Mutations as a Factor in the Production of Cancer. A Critical Review of Hansemann's Theory of Anaplasia in the Light of Modern Knowledge of Genetics." *J Cancer Res.,* 4, 181.

Wilson, E. B. (1925). "The Cell in Development and Heredity." 3rd Ed. New York: Macmillan.

Winge, O. (1930). "Zytologische Untersuchungen über die Natur Maligner Tumoren Teerkarzinome bei Mäusen." *Z f wiss. Biol.,* 10, 683.

Wintwarter, H. von. (1912). "Etudes sur la spermatogenèse Humaine." *Arch. Biol.,* 27, 91.

Woods, W. M., and DuBuy, H G (1945). "Cytoplasmic Diseases and Cancer." *Science,* 102, 591.

Woolley, G. W. (1953). "Genetics of Neoplastic Diseases." In *The Physiopathology of Cancer.,* p. 171. (Ed. F. Homburger and W. H. Fishman.) London: Cassell.

—— and Little, C. C. (1945). "The Incidence of Adrenal Cortical Carcinoma in Gonadectomized Female Mice of the Extreme Dilution Stain. 1. Observation on the Adrenal Cortex " *Cancer Res.,* 5 193.

Wollman, S. H. (1955). "Comments on the Analysis of Dose Response data in Experimental Carcinogenesis." *J. nat. Cancer Inst.,* 16, 195.

hydrochloride) it shows the intensification in the 3000 Å region due to substitution of an auxochrome at the 3-position. Addition of a drop of strong acid to the solution produces a spectrum of the 3-methoxystilbene type (cf. Figs. 6 and 7).
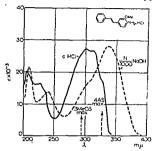
Fig. 7. Absorption spectrum of 4-amino-3-methoxystilbene hydrochloride in acid and in alkali

Some inter- and extra-molecular environmental effects

From this vast and interesting field two topics will be chosen: first examples of the differentiation of $\pi \rightarrow \pi$ and $n \rightarrow \pi$ transitions; and second, some effects of solvents, of viscosity and of temperature on $\pi \rightarrow \pi$ transitions.

Reference was made earlier to the low-frequency $n \rightarrow \pi$ structure in the spectrum of pyridine. This shoulder disappears, owing to its blue-shift, on changing from hydrocarbon to hydroxylic solvents (Fig. 8 (a), from HALVERSON and HIRT [37]), while the $\pi \rightarrow \pi$ maximum around 38,000 cm^{-1} remains stationary under these conditions. In Fig. 8 (b) is shown for comparison the pyrazine spectrum, but in this case $\pi \rightarrow \pi$ and $n \rightarrow \pi$ systems (situated around 38,000 cm^{-1} and 32,500 cm^{-1} respectively) are well separated and the $n \rightarrow \pi$ shift on changing solvents is clearer. This solvent effect on $n \rightarrow \pi$ systems has been attributed by KASHA [21] to hydrogen-bonding of the solvent with the n-electrons of the solute molecule. Salt-formation has a similar effect on $n \rightarrow \pi$ bands, for it ties down these non-binding electrons, while under the same conditions the $\pi \rightarrow \pi$ transitions shift to the red, due to the usual hyperconjugation and charge asymmetry effects [24, 37, 38]. These rules may prove useful in discovering whether a heteroatom in a ring-system has been affected, e.g. during salt formation.

PLATT [5] and KASHA [24] have pointed out further properties of these transitions, i.e. that they are weaker than $\pi \rightarrow \pi$ transitions (ϵ_{max} about 10–1000 instead of the 200–100,000 for $\pi \rightarrow \pi$ transitions), and they are easily covered by their nearest $\pi \rightarrow \pi$ neighbouring band on molecular substitution. They cannot always be detected therefore. The spectra of the azahydrocarbon, 4: 9: 10-triaza-1: 2: 5: 6-dibenzanthracene [39], shown in Fig. 9, illustrate the last point. The $n \rightarrow \pi$ transitions are hidden beneath the long-wavelength $\pi \rightarrow \pi$ system, which may be compared with

ULTRA-VIOLET ABSORPTIOMETRY

Some effects of environment on electronic spectra

E. M. F. ROE

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the sub-
stitution of perturbing groups. From the field of inter- and extra-molecular environmental
effects, two topics are chosen for discussion, i.e. the use of these effects to differentiate $\pi \rightarrow \pi$ and
$n \rightarrow \pi$ transitions, and some results of solvent, viscosity and temperature changes on $\pi \rightarrow \pi$
transitions. Some present trends in the investigation of electronic spectra are illustrated by
means of the examples selected.

A CONSIDERATION of changes in electronic spectra with changes in environment—
intramolecular, extramolecular or intermolecular—could cover a vast field of
interesting effects of extreme importance to many analytical spectroscopists.
However, since it is necessary to select material for this paper, a few examples
only will be discussed, selected, apart from personal interest, in order to illustrate
some present trends in the investigation of electronic spectra.

Fundamental processes in the absorption and emission of electronic energy

It is useful, first, to summarize the fundamental processes occurring in the
absorption and emission of electronic energy, and in doing so we shall probably
find that we use a mixed language, describing the spectra sometimes in terms of
transitions between energy levels and at other times in terms of the traditional
chromophores.

Recent advances in quantum mechanics are bringing increasing understanding
of the basic relationships between electronic energy changes in molecules and their
absorption and emission spectra. Already, for example, for dyes a number of
useful predictions of spectra and related properties can be made on the basis of
semi-empirical quantum theory. DEWAR [1] has summarized this particular field
recently; and the work of PLATT and colleagues [2, 3, 4, 5] has shown the usefulness
of quite simple theory in classifying and predicting absorption bands; (see also
BAYLISS [6], KUHN [7] and SIMPSON [8]). Such work increases the precision of our
descriptions and predictions. However, in the transitional era in which we find
ourselves in this field, the old idea of the chromophore as the absorbing or emitting
entity is still useful to the analytical spectroscopist, who still detects or estimates
these groups, normally, by their 'finger-print' bands or by the effects on these
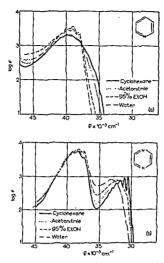bands of environmental changes.

Fig 8. Solvent effects on $n \to \pi$ and $\pi \to \pi$ absorption systems in (a) pyridine and (b) pyrazine (from HALVERSON and HIRT [37].
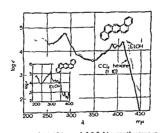

Fig 9 Absorption spectrum of 4:9.10-triaza-1·2:5·6-dibenzanthracene in ethanol (95%) and in $CCl_4 . n$-hexane (1·10). (Inset. spectrum of 1:2·5 6-dibenzanthracene in EtOH (100%).)

In Table 1 a few typical chromophores and some of their modifications are listed, with the ε_{max} and position of the longest-wavelength absorption band or band-system (corresponding to the lowest energy transition) in each case, and the solvent in which the spectrum was determined. The band-systems shown in this Table vary in origin. Thus, in some cases, as in simpler ethylene derivatives such
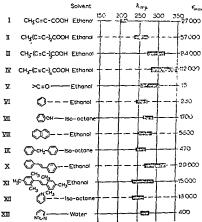
Table 1. References to data displayed

I–IV   HAUSSER K. W., KUHN R., SMAKULA A., and HOFFER M. *Z. phys. Chem.* 1935 B29 371.

V   BIELECKI J. and HENRI V. *Ber. dtsch. Chem. Ges.* 1913 46 3627.

VI   MAYNEORD W. V. and ROE E. M. F. *Proc. Roy. Soc.* 1935 A152 299.

VII   Amer. Petroleum Inst. Res. Project 44; Cat. UV Spectral Data, Serial No. 99.

VIII   As VI.

IX   Amer. Petroleum Inst. Res. Project 44; Cat UV Spectral Data, Serial No. 278.

X   BEALE R. N. and ROE E. M. F. *J. Chem. Soc.* 1953 2755.

XI   BEALE R. N. and ROE E. M. F. *J. Amer. Chem. Soc.* 1952 74 2302

XII   Amer. Petroleum Inst. Res Project 44; Cat. UV Spectral Data, Serial No. 108.

XIII   HEYROTH F. F. and LOOFNOUROW J. R. *J. Amer. Chem. Soc.* 1934 56 1728.

[Note that the position of a band in the Table is represented diagrammatically by a block whose wavelength-limits are those at the half-ε_{max} value of the band]

as I–IV (Table 1) it is known that the band corresponds to a single electronic transition (HAUSSER *et al*, [9] and POTTS [10]) which is strongly allowed, i e. one whose initial and final energy states have such properties that they combine and there is a high probability that the transition will occur (cf. reference [11]). The ε_{max}

the corresponding absorption in the parent hydrocarbon (inset to Fig 9); and in both spectra a red shift occurs on moving from hydrocarbon to hydroxylic solvents.

In accordance with KASHA's further generalization [21], this azahydrocarbon. showing π-electronic states as its lowest singlet states, is fluorescent. while compounds with clear $n \rightarrow \pi$ low-frequency absorption in general are *not* fluorescent. For KASHA has pointed out, from a study of the luminescence properties of molecules. that if $n \rightarrow \pi$ absorption falls at lower energy than any $\pi \rightarrow \pi$ transition, the molecule does not fluoresce, as is observed in all aliphatic ketones and many aromatic ketones, in aldehydes, azo-compounds. quinones and nitro-compounds and in most simple N-heterocyclics. Again, this generalization can be useful in analytical work. Further, since $n \rightarrow \pi$ transitions are weaker than corresponding $\pi \rightarrow \pi$ transitions, the $n \rightarrow \pi$ excited states are much longer-lived than analogous $\pi \rightarrow \pi$ excited states, in accordance with the classical inverse relation between mean life-time of an excited state and the intensity of the corresponding absorption band [21, 40]. A long intrinsic life-time for the excited state is probably of the utmost importance for the utilization of excitation energy [21] Like triplet states. therefore, these $n \rightarrow \pi$ singlet excited states may have some importance in energy transfer processes, and it is possible, for example, that they have not been considered sufficiently in relation to the biological role of the proteins and nucleotides and their derivatives, $n \rightarrow \pi$ excited states will occur in these under certain conditions and in certain environments

The question of solvent, temperature and viscosity effects in $\pi \rightarrow \pi$ absorption systems will be limited to some extent by omitting any discussion of complex formation. It will be assumed that only *physical perturbation* of solute molecule by solvent occurs, so that every solute molecule is similarly perturbed and does not have equilibrium between two distinct solute species one free and another and one involved in complex formation in the chemical sense of saturation of the perturbation by an active solvent molecule (BAYLISS and BRACKENRIDGE [41]).

An old generalization which may be recalled and which holds for a nonpolar solute molecule in a non-polar solvent, is KUNDT's rule [42]—that band-shift to the red occurs increasingly with increasing refractive index of solvent. There is a fairly clear theoretical basis for this limiting case [43], but the more usual variations in these conditions still need clarifying, as when polar solute and/or solvent molecules are introduced. It has been pointed out, further, that the intensity of a band should change approximately in relation to its red-shift, on changing solvent, and this generalization appears to hold in a number of cases (BAYLISS [44]). Overlap of transitions will obscure these effects, however, and the fine detail in a band-envelope may have its own interest. The spectrum of stilbene will be used again to illustrate these and other points.

Further examination of Fig. 2 shows that the stilbene spectrum changes with the solvent, but as with some other classes of compounds, the refractive index law is not obeyed without qualification. The spectrum in ethanol lies slightly further to the red than that in n-hexane. although the refractive index of the latter is a little higher. Benzene, with the highest refractive index of the three solvents shown, gives the largest red-shift, but in addition, an interesting change in the

and the area under the band envelope ("absorption strength") were shown [9] in these compounds to be proportional to the number of double bonds in the polyene chain. just as later empirical relationships have been observed between the position of the first absorption maximum and the chain length in, for example, the cyanine dyes, a relationship which theory now can explain fairly simply [1].

Compared with that for the —C≡C— group, the longest-wavelength band given by the >C═O chromophore (V, Table 1) is very weak, corresponding to a transition which is formally forbidden (McMurry [12]). Symmetry considerations show that the states concerned should not combine and the low extinction coefficient is evidence of this, as in the next example, benzene (VI, Table 1; see Sponer et al. [13]). However, distortion of the molecular symmetry by molecular vibrations removes this forbiddenness. and this longest-wavelength transition appears. although with low probability and thus with low intensity [13, 14]. The situation is quite changed when a typical auxochrome, or colour-enhancing group, such as a hydroxyl is substituted in the benzene ring, completely changing its symmetry (VII, Table 1). The long-wavelength band appears further to the red; it increases in intensity. and the vibrational structure which is found in the benzene spectrum is much reduced.

The annelation of successive benzene rings, beginning with naphthalene (VIII, Table 1), changes the spectrum even more, although Platt's theoretical treatment 2, 3, 4] has led to a considerable degree of understanding of the spectra of the condensed ring hydrocarbons, and such work has been extended by other investigators; (see, for example, [1, 15, 16]. In these aromatic compounds, the mobile. unsaturation π-electrons concerned in these transitions have fairly free passage over the molecular skeleton and the whole molecule has to be regarded, rather crudely, as the chromophore. In diphenylmethane (IX, Table 1) where a —CH₂— group is interposed between the two benzene rings, this free passage for the π-electron charge is interrupted. The long-wavelength band remains in about the benzene (or toluene) position and has approximately twice the molecular extinction coefficient of the latter, corresponding to virtually independent absorption by the two halves of the molecule.

Stilbene, having two benzene rings linked by the —C═C— group (X, Table 1) shows no evidence in its long-wavelength system of the original benzene bands. The former, apparently, corresponds to a transition involving the complete molecule, but having vibrational structure originating in the —C═C— stretching frequency (cf. Beale and Roe [17]). Whether any of the original benzene transitions are hidden in the envelope of this intense band is a point on which some evidence will be offered below. However, it is known [18, 19] that when conjugation between the separate parts of the molecule is hindered sterically by the substitution of methyl groups at appropriate positions (i.e. 2,6,2′ and 6′ in the phenyl groups) as in the hexamethylstilbene XI (Table 1) the absorption again changes, reverting to a spectrum more like that of a simple benzene derivative or of diphenylmethane.

The last two chromophores in Table 1, pyridine (XII) and pyrimidine (XIII), are of interest in many biological investigations, and they exemplify a type of

vibrational structure of the band may be observed, in that the β-peak rises compared with the γ-peak as the band-system shifts to the red, as does the long-wavelength shoulder, α. In fact, it is found that the β-peak can be taken as one index of the band and its frequency seems to vary with refractive index in the two classes of solvents if hydroxylic solvents are separated from the rest. The shift to the red increases. e.g. for the series:—mixed MeOH : EtOH, 95% EtOH, 50 : 50-glycerol: EtOH, to 99½% glycerol : EtOH as solvent, as also in a series of non-hydroxylic solvents of increasing refractive index, varying from mixed isopentane : methyl-
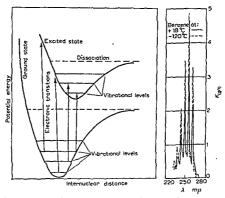
Fig. 10. Schematic potential energy curves for ground and excited states in a simple molecule, with absorption spectrum of benzene (in mixed alcohols) at +18°C and −120°C

cyclohexane through chloroform to benzene. Now, in the case of a non-polar solute such as stilbene, having no dipole moment, dispersion forces only are expected to act in solvent-solute interaction, even with a polar solvent, and the main solvent effect should be the polarization red-shift depending on solvent fractive index, unless the solvent molecules in any way constrain any part of the molecule (cf. BAYLISS [43]). It seems from the data summarized below* that such constraint can operate in the case of stilbene, and that this is the reason for the slightly greater red-shift in hydroxylic solvents than in non-hydroxylic solvents, accompanied by a regular change in the relative intensities of the vibrational peaks.

However, temperature changes also give interesting results in this connection. It is well known that changes in detail may be observed in electronic absorption bands with changing temperature. As the temperature falls, higher energy

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* A detailed discussion of these data is being prepared for publication.

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right kidney. Sometimes it is even necessary to cut an adherent suprarenal off the kidney or the liver by means of diathermy.

In itself the operation is not dangerous provided the operator is familiar with the normal and abnormal arterial supply and venous drainage of the suprarenal glands, so that these vessels may be caught, divided and tied or coagulated with the diathermy before the bleeding obscures the view at the bottom of the deep wound. The most important vessel to secure is the central vein from the right adrenal which, after a course of variable length, empties directly into the inferior vena cava. Other vessels are derived from the inferior phrenic and renal vessels and occasionally from the aorta. If the right suprarenal is involved with a metastasis, it is sometimes impossible to remove it without damaging the inferior vena cava. The operator therefore should be prepared to repair tears in that vessel. I always have available a Tom Smith clamp and an arterial suture for use should such an accident occur. Franksson and Hellström (1956) state that the vena cava was injured in 11 out of their 118 adrenalectomies.

Alternative combinations of the different steps in the operation are commonly practised. For example, as in the case cited above, bilateral oöphorectomy may be done early in the course of the disease and both suprarenal glands removed later at a one-stage operation. Some prefer the patient to lie first on one side and then on the other, others to place the patient on her face so that both suprarenals can be removed without having to move the patient. Others prefer a long transverse anterior incision, the suprarenals being removed from the side. In itself the operation of bilateral adrenalectomy is not dangerous provided cortisone is administered intelligently and the blood pressure is maintained. The operative mortality is in direct proportion to the extent of the metastases. By careful selection the operative deaths can be kept to a minimum, but unfortunately metastatic carcinoma does not allow of careful selection and often the best surgeon will take the greatest risks and will have the highest death rate, but he may do the greatest amount of good.

Worth-while remission has been achieved in about 50 per cent. of patients who have had this operation. Cade, in his Hunterian lecture in 1954, found that 52 per cent. of his patients showed improvement, in some cases remarkable. Dao and Huggins, in 1955, found that 41 per cent. out of 175 patients obtained relief. At the Royal Marsden Hospital we have found the figure to be almost exactly 50 per cent. Many of these patients have been desperately ill, e.g. in deep coma, and a few have been refused hypophysectomy.

HYPOPHYSECTOMY

Removal of the pituitary for metastasizing carcinoma of the breast was introduced by Olivecrona in 1951. It is done through a right temporal flap, the pituitary being approached by retraction on the right optic nerve. The stalk is divided. The diaphragma stella is torn open and the pituitary removed piecemeal. Hæmorrhage may be difficult to control. It is a more

difficult and dangerous operation than adrenalectomy and probably in its[...]
carries a mortality of about 4 or 5 per cent.

Alternative methods of ablating the pituitary have been devised by Forre[...]
and Peebles Brown (1955), who implanted radon seeds into the pituita[...]
through the nose under x-ray control; and by Greening (1957), who similar[...]
implants pellets of radioactive gold or yttrium. Stevenson (1957) has in[...]
proved the radiological method of controlling the position of the seeds [...]
using an image intensifier. A dose of 70,000 rœntgen units of gamm[...]
irradiation appears to be necessary to cause necrosis of the pituitary glan[...]
Unfortunately, the operative mortality is not reduced by adopting the[...]
relatively minor, ingenious procedures because a proportion of the patien[...]
perhaps 6 to 8 per cent., die of meningitis owing to the fact that the radi[...]
active source is introduced through the nose.

The results obtained by hypophysectomy are perhaps slightly more sat[...]
factory than those obtained by bilateral oöphorectomy and adrenalectom[...]
although the operative mortality is higher and the complication rate (e[...]
blindness, personality changes) is greater. Atkins, Falconer, Hayward a[...]
MacLean (1957) have compared the two methods by a process of reckoni[...]
a mean clinical value (Walpole and Paterson, 1949) on an assessment of t[...]
state of the metastases. They reckon that the over-all results of hyp[...]
physectomy 'seem to be superior to those of adrenalectomy with oöph[...]
ectomy, but the results are not statistically significant'. It is interesting[...]
note that all reports on pituitary fossæ examined post mortem after orthod[...]
hypophysectomy have shown that the majority, even of the successful cas[...]
contain fragments of viable pituitary tissue, suggesting that a good res[...]
following hypophysectomy can be obtained even when some of the gla[...]
is left behind. Stretton Young (1957) has examined by serial section t[...]
pituitary fossæ of patients who have had radioactive implants and has fou[...]
that sometimes only a small proportion of the pituitary is destroyed. T[...]
results are less uniform.

SUCCESS OR FAILURE

Since success or failure depends primarily upon the tumour being hormo[...]
dependent, a preoperative test providing a reliable guide on this point wou[...]
be of inestimable value, but none has yet been devised. Hadfield (19[...]
suggests that a high level of mammotrophic hormone present in the ur[...]
of women suffering from inoperable carcinoma of the breast is probab[...]
indicative that endocrine surgery will be beneficial. Up to now and probab[...]
for some time to come, it will be necessary in every case to 'try the expe[...]
ment' on each patient.

Apart from hormone dependence, adrenalectomy will fail if there a[...]
accessory rests of adrenal tissue remote from the gland. These have som[...]
times been discovered post mortem as low down as at the bifurcation of t[...]
aorta. In some cases, possibly other organs take over the manufacture [...]
œstrogens. It is in this group of failed adrenalectomies that subsequ[...]

vibrational transitions are reduced in probability and solute-solvent interaction is reduced, and thus the blurring of the band envelope due to the multitude of vibrational transitions is reduced, and peaks normally appear sharper at lower temperatures. This sharpening effect is fairly general, although it varies in degree with the type of molecule and transition concerned. A typical example is benzene [45], shown on the right in Fig. 10, together with the type of potential energy curve [46] which is applicable to such a case at room temperature. The detailed changes in the curve with temperature will not be discussed (cf. reference [11] p. 294 for further discussion) but an obvious result is the sharpening of the structure, with no frequency changes. It is probable that the only change in the potential energy curves at low temperature is the introduction of pseudo-potential humps near the dissociation limit, so that the fragments of the molecule cannot drift apart as readily but there will be no effect on the Franck-Condon principle and the nearest excited state vibrational levels involved in the room temperature transition will open out at low temperatures also.

In Fig. 11, the spectrum of stilbene is shown in three solvent mixtures at room temperature and at low temperatures. A similar sharpening is observed in all three solvents in both low-frequency band-systems. A and B, although their peaks are sharpened more than γ in all cases, and there clearly is a shift of area from the high-frequency side of band A into α and β. (Changes in A are not accurately determined than in B or C, because A is more easily separated from its neighbouring band, B.) There is no change in band-intensity with temperature in transition A^*. For example, in mixed alcohols (Fig. 11c) f is 0·728 at $+20°C$ and 0·737 at $-146°$, a change of just over 1%, which is within the experimental error. In fact, the change in total f for the two bands $(A+B)$ is also only about 2%. Nor is any significant frequency-shift observed if the low-frequency slopes of bands A and B are compared at the two temperatures; it is the peak frequencies which change, owing to the changing overlap of the vibrational bands with changing intensity distribution on temperature reduction. Similar results are obtained in all three solvents, although in the mixed hydrocarbons (Fig. 11b) at $-185°C$ the peak sharpening is not quite as great as in alcohols at $-146°$, and in glycerol (Fig. 11c) the differentiation is greater. Even at room temperature, in the latter case, there is an increase in the peak ratios $\frac{\varepsilon_\alpha}{\varepsilon_\beta}$ and $\frac{\varepsilon_\beta}{\varepsilon_\gamma}$ and a red-shift, compared with ethanol; and at $-61°$ the peak changes are approaching the result reached in the hydrocarbon solvent at $-185°$.

It is worthwhile to examine the differences between the three hydroxylic solvents used for the spectra in Fig. 11(c). First, the refractive index of the solvents increases in the order 95% EtOH, glycerol at $+20°$ and glycerol at $-61°$. However, n is even greater in benzene than in glycerol at $+20°$, yet the changes in ε-ratios are not as large as in benzene solution. Secondly, viscosity also increases in the order of the refractive index increases above, but it has been found that at $+20°$ in a non-hydroxylic low-molecular weight polymer (polyisobutene) of

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* This is in contrast to an earlier published result [17] which was found recently to be subject to experimental error.

THE ACTION OF ALKYLATING AGENTS ON DEOXYRIBONUCLEIC ACID AND GUANYLIC ACID

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The action of the nitrogen mustard HN2 (di-(2-chloroethyl)methylamine) on deoxyribonucleic acid (DNA) from calf-thymus at pH 7 and 37°C. (0·4 moles of HN2 per nucleotide) results in a change in shape of the absorption curve due to DNA, indicating that alkylation of purine or pyrimidine rings occurs, at a rate at least comparable with that of the possible alkylation of phosphate groups.

Reactions with nucleotides suggested that the guanine moiety of DNA was most susceptible to alkylation. This was confirmed by acid-hydrolysis of DNA. Paper chrom... ... ... ratio guanine:

0·3 (control, 0 · · ·   · · ·
principal ($R_F = · · ·$, Wyatt's solvent) having the absorption spectrum $\lambda_{max} = 280$, 248 (0·8) (for all spectra, the solvent was aqueous buffer pH 7, wave-lengths are in m... and th... · · · · · · relative extincti · · · · ·
if present). Th ... · · · · ...... of the alkylated DNA also showed absorption of this type, whereas no purines dialysed from the control, suggesting that alkylation may render guanine of DNA more susceptible to hydrolysis.

Comparison of this absorption spectrum with those of alkylated guanines shows its resemblance only to that of a 7-alkylguanine (cf. 7-methylguanine,[2] $\lambda_{max} = 282$, 246 (0·86)). This would imply that the primary product of alkylation is of the type of a quaternary salt. Support for this has been obtained by comparing the alkylation of 1 : 7-dimethylguanine with that of guanylic acid. From the silver derivative of 1 : 7-dimethylguanine a methiodide was prepared,[3] having high mobility to the cathode in paper electro-phoresis, and absorption spectrum, $\lambda_{max} = 281$, 255 (1·5). Methylation of guanylic acid (1 mole Me_2SO_4, pH 7, 37°C.) gave three products separable chromatographically, the principal having half the mobility of guanylic acid in electrophoresis at pH 6·1, and shown to contain ribose by the aniline phosphate colour reaction. Their absorption spectra were similar in type (here designated M1), and similar to that of I : 7-dimethylguanine methiodide, e g. for the principal product $\lambda_{max} = 280$, 254 (1·1).

In alkaline solution at 23°c both the M1 products and the quaternary salt underwent a ... and irreversible change to products having spectra w... a single peak, near 270 mμ (type M2). The rib... ... ... was retained Similar products from the ... ... action of ethylene and propylene ox... ... in guanosine in unbuffered aqueous solu ... ... have been isolated.

On acid hydrolysis (N HCl ... ... 100 c) the M1-type products yield... ... ... with $R_F = 0·34$ (Wyatt's solvent) ... ... 246 (0·9). The spectrum is close to that ... ... for 7-methyl-guanine, although an authentic spec... ... of this has not yet been compared It seems ... ... that the M1 products are 7-methylgua... ... and that M2 products are ribotides or ribosides the structures of which are at present unknown although they are likely to be corresponding carbinols or their rearrangement or ring-fission products

We thank Prof. J. A. V. Butler for the supply of DNA. The work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

C. A. W. holds the Gordon Jacobs Research Fellowship, Royal Marsden Hospital.

References
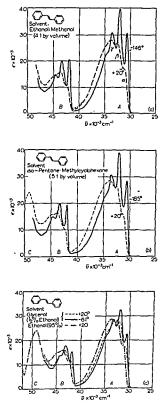1 Wyatt, P... ... ...

Fig 11 Effects of temperature and solvents on the absorption spectrum of *trans* stilbene

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The Introduction and Breeding of the Chinese Striped Hamster (Cricetulus griseus) in Great Britain

By C. SMITH (Chester Beatty Research Institute) London

The Chinese Striped Hamster (*Cricetulus griseus*) first became known as a laboratory animal in 1919, when Hsieh (1919) substituted it for mice. At that time pneumonia was very prevalent in Peking, and extensive laboratory tests were necessary to identify the various types of pneumococcus. For that purpose the Chinese Striped Hamster was found to be an excellent laboratory animal. In China the species is widespread and the supply sufficient to meet the local demand for laboratory studies. Interest in this hamster, however soon increased in laboratories, both in China and Great Britain, when it became known by the work of Smyly and Young (1924) and Hindle and Patton (1926) that this animal is an excellent host for the parasite *Leishmania donovani*, the cause of Kala Azar. Since then various efforts have been made to breed the species in captivity. Hindle brought back to England a certain number of these hamsters in 1927, and some time later his assistant, Dr. Feng Lan Chow, brought a further consignment to this country but they did not succeed in breeding from them. However, the late Professor P. A Buxton, of the London School of Hygiene and Tropical Medicine, obtained a litter from a pair of hamsters belonging to this stock and this would seem to have been the first successful mating in captivity (personal communication) Only one litter was born and in later years Buxton failed to breed them. Similarly, Chang and Wu in Peking (1938) obtained a few litters in 1938, but further efforts to maintain their hamster colony were unsuccessful

It was of considerable interest to cytologists when Pontecorvo (1944) reported that the Chinese Striped Hamster has the lowest chromosome number in mammals outside the Marsupials He made use of Bouin fixed material collected by Professor E. Hindle, of the University of Glasgow, during his expedition to China in 1925-26 Owing to this new interest it was therefore fortunate that at the Tumblebrook Animal Farm, in New Jersey U S A . Schwentker succeeded in breeding the species and offered them for sale From his stock a few animals of both sexes were obtained by the Chester Beatty Research Institute in London in 1950 and from them we were successful in rearing 13 litters The colony, however, had to be kept small (about 50) owing to

lack of space and breeding was discontinued

From Schwentker's stock of Chinese Striped Hamsters Dr. G. Yerganian obtained a few animals and by great patience overcame the difficulties of breeding and established a very large colony at the Children's Hospital, Boston, U.S.A. In 1954, the Chester Beatty Research Institute obtained a further supply from Dr. Yerganian and now these have been built up into a large colony. Subsequently a flourishing new colony has been established at Harwell by Bracken from Chester Beatty stock; recently another colony has been started with 50 animals at the Institute for Medical Research, Mill Hill.

The Chinese Striped Hamster is smaller than the Golden Hamster (*Mesocricetus auratus*), sometimes erroneously termed Syrian Hamster. Its length is approximately 9-10 cm. from muzzle to the base of the tail, and the weight of mature males averages about 35 grammes and females 25 grammes. The fur is rather short with a silky gloss and agouti in colour. There is a narrow black streak down the middle of the back and the belly is white The males have a large distended scrotum, about 3 cm. long. Both sexes have cheek pouches in which they store food.

The adult animals are kept singly in metal cages (9in × 6in. × 4in.) except for breeding, when a male and female are put together in a large box (28in. × 14in. × 4in). Feeding is simple because they are tolerant of a widely varied diet including green vegetables. In 1931, Parkes, of the Institute for Medical Research, London, studied the oestrus cycle of this hamster and found that its duration was slightly more than 4 days In our colony, the oestrus cycle is somewhat shorter, rarely reaching 4 days and more often only 3½ days The duration of the "heat" period, the only time when successful copulation is possible, is only about 2 hours. The gestation period is 20 days and the young at birth weigh only 1-2 grammes Fur begins to grow when they are about 4 days old and the young are completely covered with hair by the 7th day Their eyes open on the 12th day and then they begin to feed on their own They are usually weaned when 24 days old The size of litter varies, from 3 to 7, the most common litter size being five It is advisable to separate litter mates before they reach the age of 8 weeks, because

macroscopic viscosity greater than that of glycerol the changes in ε-ratios again are not as large as in glycerol. The "microscopic" or "local viscosity" of the glycerol solution, referred to a single stilbene molecule would be greater, however, than in polyisobutene, due to association of the glycerol molecules. From the results quoted so far, it appears that the change in shape and redistribution of intensity in stilbene's *A* and *B* bands, compared with its spectrum at room temperatures in a hydrocarbon such as *n*-hexane, can be produced to greater or lesser extent by (a) using a hydroxylic solvent, particularly (as in glycerol) one in which
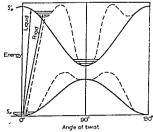
Fig. 12. Variation of potential energy with twist of the double bond in
ethylenic compounds (redrawn from KASHA [21])

association and therefore high 'local' viscosity occurs; (b) by dissolution in benzene and (c) by reducing the temperature in hydrocarbon or hydroxylic solvents, so as again to increase 'local' viscosity. Increase in macroscopic viscosity, as when polyisobutene at room temperature is used as solvent, does not have the above effects. The reason for these results probably lies in the particular potential energy curves which require to be considered for a molecule such as stilbene (Fig. 12.)

Thus, in ethylenic compounds, the twisting motion about the double bond has considerable effect on the shape of these curves. MULLIKEN [47] and MULLIKEN and ROOTHAAN [48] have discussed the electronic-vibrational interactions in ethylene, and KASHA [21] has considered the changes envisaged in complex ethylenic molecules in terms of a potential diagram such as that given in Fig. 12. Transitions occur in the freely twisting molecule to various vibrational levels of the excited torsional potential (S'_a in Fig. 12), as in the transition marked 'liquid' or in the vapour. In stilbene, however, it is considered that the phenyl groups may be large enough to inhibit the torsional mode of vibration, so that the potential curves may change shape (as in the dotted curves, Fig. 12), and the pseudo-potential humps introduced, particularly in the excited state, would cause excitation to occur to a nearly equilibrium configuration, in a rigid solvent. This change should result in a redistribution of intensity among the vibrational transitions, according to the Franck-Condon principle, and the low-temperature spectral changes show this quite clearly. The experimental results quoted above suggest

of their aggressive behaviour to each other when kept in the same cage. In our colony the hamsters became mature only after 12 to 16 weeks, males maturing earlier than females, but by providing extra facilities for exercise in the cages, Yerganian was able to breed from 8 weeks old females.

The cause of previous failures in breeding the Chinese Striped Hamster in captivity seems to have been due to the fact that the time of oestrus or "heat" period can only be deter-

Chinese Hamster

mined with difficulty. If a male is placed together with a female which is not at this period, mating may be attempted but the effort of the male not only fails, but also provokes savage fighting between the pair. When a female is in oestrus, thus ready for mating, she is placed in a large breeding box together with a male. As a rule the female then approaches the male, sniffs and runs away. The male having scented the female on heat, runs after her. The female stops suddenly and humps up her back (lordosis). Then the male mounts her and copulation takes place. Both sexes remain apart after copulation for a brief period but resume activities by repeating the same behaviour as described above. The mating pair can be left together for 2-3 hours during which period coitus usually takes place several times (up to 20). The male must be removed as soon as no more coital play is observed. The sexes must also be separated if a "copulation-plug" is observed in the orifice of the vagina. Though the behaviour of the male and

female and the presence of a copulation-plug indicates successful coitus, mating should be attempted again at the next oestrus period. If the female and male behave aggressively to each other and fight, it can be assumed that the first mating was successful.

The Chinese Striped Hamster is nocturnal and its sexual activity is confined to darkness In earlier attempts at breeding, mating trials were carried out in the late evening In order to facilitate the separation of mating pairs before fighting started between males and females, Y-- --- - ' ' ' .
scheme,
during d. .
system was introduced and now the Hamster are kept in the dark from 1 p.m to 10 p m and illuminated for the remainder of the 24 hours, thus having 9 hours darkness and 15 hours daylight. Under these circumstances animals in oestrus can easily be mat d during the ordinary working hours without the further inconvenience of keeping them und   of   own tion during the night. With this   procedure numerous young have been bon and it have been successful in building up large colony of these hamsters.

Acknowledgments

My thanks are due to Dr E Hindle f R S and to Professor P. C Koller, for their interest and help.

References

Chang, C. Y. & Wu, H (1938) Growth and reproduction of laboratory bred hamster Cricetulus griseus. Chinese J. Physiol. 13, 109.

Hindle, Edward & Patton, W. S. (1926). Experiments bearing on the Susceptibility of the Striped Hamster (Cricetulus griseus) to Leishmania of Chinese Kala Azar. Proc. Roy. Soc. B. 100, 374.

Hsieh, E. T. (1919). A new Laboratory Animal, Cricetulus griseus. Nat. Med. J. China. 6, 20.

Yerganian, G. (1955). The Striped-back or Chinese Hamster (personal communication).

Parkes, A. S. (1931). The Reproductive Processes of certain Mammals.   Part I. The Oestrus Cycle of the Chinese Hamster (Cricetulus griseus). Proc. Roy. Soc. B. 108, 138.

Pontecorvo, G. (1944). Meiosis in the striped hamster.   Proc. Roy. Soc. Edin. B. 62, 32 .

Smyly, H. J. & Young, C. W. (1924). The Experimental Transmission of Leishmania to Animals. Proc. Soc. Exp. Biol. & Med. 21, 354.

that the local, microscopic viscosity is also an important factor in these phenomena (although not the macroscopic viscosity) and that other factors may operate in the same direction. Thus, an increase in the mass of the phenyl groups in stilbene through solvation, as is conceivable, particularly in the excited state, in a hydroxylic solvent, could restrict the freedom of torsion about the double bond and initiate the changes in the shapes of the potential curves which lead to redistribution of intensity. This could happen in benzene solutions also, through van der Waals-London forces. Other evidence in this connection must await a detailed publication. However, support for this interpretation is to be found in the spectrum of stilbene vapour. STYLE [49] has found almost no structure in this vapour spectrum (at about 140°C and six pressures between 1·48 and 1·43 mm Hg), and clearly this result would be expected in the torsional potential case when, as in the vapour, freedom of twist about the double bond is unrestricted.

Conclusion

Some examples have been given of the effects of environment on electronic spectra, illustrating just a few of the present trends in this field which are worth further investigation. These may be summarized:

(1) Examination of low-intensity $n \rightarrow \pi$ systems and $\pi \rightarrow \pi$ triplet transitions which, because of their low intensity and consequent long lifetimes, may be important in energy transfer phenomena. The absorption in the tail of a spectrum is not to be disregarded.

(2) Awareness of possible complexity in an apparently simple spectral outline on account of overlap of both electronic and vibrational transitions may lead to the use of environmental changes to resolve this structure, e.g. by solvent or temperature changes or by chemical substitution, although the last may be more difficult to interpret.

(3) An interest in band-intensity measurements (f-values) is of increasing importance throughout the field of electronic spectroscopy both in absorption and emission. Possibly this is one of the strongest reasons for the practical use of a frequency scale rather than wavelengths in measuring and plotting electronic spectra.

References

[1] DEWAR M J. S. *Chem. Soc Special Publication No.* 4 1956 64.
[2] KLEVENS H. B. and PLATT J. R. *J. Chem. Phys.* 1949 **17** 470.
[3] PLATT J. R. *J. Chem Phys.* 1949 **17** 484

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Glutamic Dehydrogenase in the Developing Chick Embryo

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The processes of morphogenesis and differentiation in embryos may be expressed on the molecular level as an increasing complexity of arrangement of proteins in the embryonic cell. This arrangement may be accompanied by development or loss of certain enzymic activities during differentiation until the complement of enzymes characteristic of adult tissue is attained. Boell (1955) has reviewed the considerable amount of evidence that enzyme development is linked with functional differentiation during development

The ontogenic sequence of carbohydrate and protein as primary energy sources during develop-

ment of the chick embryo (Needham, 1931) may be accompanied by the appearance of enzymes concerned with the conversion of carbohydrate into protein precursors. Glutamicdehydrogenase (GDH) plays an interesting role in amino acid metabolism, by providing a possible pathway for the production of glutamic acid from carbohydrate sources by the fixation of ammonia, or as an 'L-amino acid oxidase' in conjunction with the transaminases (Braunstein & Bychkov, 1939; Braunstein & Azarkh, 1945; Meister, 1955).

GDH activity has been measured in blastoderms, embryos and their extra-embryonic tissues during

[4] PLATT J. R.   *J. Chem. Phys.* 1950 18 1168

[5] PLATT J. R.   *J. Chem. Phys.* 1951 19 101

[6] BAYLISS N. S.   *J. Chem. Phys.* 1948 16 287

[7] KUHN H.   *J. Chem. Phys.* 1949 17 1198

[8] SIMPSON W. T.   *J. Chem. Phys.* 1948 16 1124

[9] HAUSSER K. W., KUHN R., SMAKULA A.   *Z. Phys. Chem.*

[10] POTTS W. J.   *J. Chem. Phys.* 1955 23 65

[11] HARRISON G. R., LORD R. C., and LOOFBOUROW J. R.
     Hall, Inc., New York, 1948, p. 216

[12] McMURRY H. L.   *J. Chem. Phys.* 1941 9 231 241

[13] SPONER H., NORDHEIM G., SKLAR A. L.

[14] HERZBERG G. and TELLER E.   *Z. Phys. Chem.*

[15] CRAIG D. P.   *Rev. Pure Appl. Chem.*   2, 5

[16] MOFFITT W.   *J. Chem. Phys.* 1954 22 320

[17] BEALE R. N. and ROE E. M. F.   *J. Chem. Soc.*

[18] HADDOW A., HARRIS R. J. C., KON G. A. R.
     1948 A241 147.

[19] BEALE R. N. and ROE E. M. F.   *J. Amer. Chem. Soc.*

[20] MULLIKEN R. S.   *J. Chem. Phys.* 1939 7 364

[21] KASHA M.   *in Luminescence of Biological Systems*
     Sci. 1955, ch. 2.

[22] CRAIG D. P. and ROSS I. G.   *J. Chem. Soc.* 1954 1589

[23] LEWIS G. N., LIPKIN D., and MAGEL T. T.   *J. Amer.*

[24] KASHA M.   *Disc. Faraday Soc.* 1950 9 14

[25] COULSON C. and JACOBS J.   *J. Chem. Soc.* 1949 1983

[26] BEALE R. N., LINDERMAN A., and ROE E. M. F.

[27] BEALE R. N. and LINDERMAN A.   *J. Chem. Soc.* 1953

[28] MULLIKEN R. S.   *J. Chem. Phys.* 1939 7 121

[29] MULLIKEN R. S. and RIEKE C. A.   *Rep. Prog. Phys.*
     London, 1941, Vol. 8, p. 231.

[30] KUHN W.   *Helv. Chim. Acta* 1948 31 1780

[31] PLATT J. R.   *J. Amer. Chem. Soc.* 1952 74 2350

[32] BEALE R. N. and ROE E. M. F.   *J. Phys. Chem.* 1957

[33] WENZEL A.   *J. Chem. Phys.* 1953 21 403

[34] LONGUET-HIGGINS H. C. and MURRELL J. N.   *Proc. Phys. Soc.*

[35] MURRELL J. N.   *Proc. Phys. Soc.* 1955 68A 970

[36] HEILBRON F.   *Chem. and Ind.* 1954 984

[37] HARTLEY G. S. and HIRT R. C.   *J. Chem. Phys.* 1951 19 711

[38] McCONNELL H.   *J. Chem. Phys.* 1952 20 700

[39] OSBORNE T. and TIMMIS G. M.   *J. Chem. Soc.* 1952 4345

[40] LEDERER E. R.   *Bull. Soc. Chim. biol.* 1914 16 767

[41] BAYLISS N. S. and HULME L.   *Austral. J. Chem.* 1953 72 3372

[42] KENNY A.   *Rev. Phys.* 1951 1674 674

[43] BAYLISS N. S. and McRAE E. G.   *J. Phys. Chem.* 1954 58 1002

[44] BAYLISS N. S.   *J. Chem. Phys.* 1950 18 292

[45] MAYER J. R. W. A. and BRODE W. R.   *Proc. Roy. Soc.* 1937 A155 601

[46] BRAUDE E. A.   *Chemical Reviews of Light Absorption* Jones 1945 45 1144

[47] PEIERLS H.   *Phys. Rev.* 1953 81 370

[48] MULLIKEN R. S. and ROTHENBERG C. A.   *J. Chem. Phys.* 1951 83 269

[49] PORTER D. W. J.   *Private communication*

Discussion

development of the chick embryo, and the levels of enzyme activity compared with those of the 9-week-old hen. A preliminary report of this work has been presented (Solomon, 1956).

EXPERIMENTAL

Materials. α-Oxoglutaric acid (British Drug Houses) was recrystallized twice from hot acetic acid. Reduced diphos phopyridine nucleotide (DPNH) and a solution of purified ox-liver GDH were supplied by C. F. Boehringer and Soehne, Mannheim.

Tissues. Eggs were obtained from Rhode Island Red × Light Sussex fowls (Agricultural Research Council, Compton, Berkshire), and were incubated at 37° in a Westernette

logical development was classified according to the stages proposed by Hamburger & Hamilton (1951). The average incubation times quoted by these authors for each stage were used so that morphological development is expressed as 'corrected incubation time'. Blastoderms were removed whole until 35 hr. (10 somites stage), when the opaque area was dissected from the embryo. When the opaque area became vascular, it was subdivided into the vascular area (limited by the *sinus terminale*) and the outer periphery of the yolk sac, until the vascular area completely surrounded the yolk (at about 7 days of development). In later stages the total extra-embryonic tissue was separated into the yolk sac and allantoic membrane The tissues studied were the blastoderm (embryo with opaque area), the embryo from 35 hr., the vascular area (35 hr.–6 days), total yolk sac (from 35 hr.) and the allantoic membrane from 6 days of incubation. Tissues were stored at −15°.

Assay of enzyme activity. The method used was essentially

decrease in optical density at 340 mμ. The reaction mixture contained 1·6 μmoles of KCN, 1·2 m-moles of nicotinamide, 1·5 μmoles of DPNH (sodium salt), 0 1 M phosphate buffer, pH 7 6 (2·5 ml.), 60 m-moles of NH₄Cl and 0 1–0·2 ml. of enzyme solution or homogenate in each cuvette; total volume was 3 ml. Measurements were made in a spectro-photometer (Unicam SP, 500) at room temperature (20°). The optical-density reading for the blank was set at 0 4, and

made in duplicate. Hogeboom & Schneider (1953) have shown with mouse-liver homogenates that the amount of DPNH oxidized under similar conditions corresponded stoichiometrically with the amount of glutamic acid formed. One unit of GDH activity is defined as that amount which causes 1 μmole of DPNH to be oxidized in 1 min., 6 22 × 10⁶ (Horecker & Kornberg, 1948) being used as the molecular extinction coefficient of DPNH.

Protein determination. The method of Sutherland, Cori, Haynes & Olsen (1949) was used. Bovine plasma albumin (Armour Laboratories) dissolved in 0·1 M-phosphate buffer, pH 7 6, was used as standard protein. Estimations were made within the range 40–200 μg. of albumin in 5 5 ml

pH optimum. All assays were made in 0 1 M-phosphate buffer, pH 7·6, which was found to be the optimum pH of GDH in an homogenate of 9-day-old chick embr s.

Homogenates Copenhaver, McShan & Meyer (1950) and Hogeboom & Schneider (1953) found that GDH activity was greater when tissues were homogenized in water instead of in isotonic sucrose solutions All homogenates in this work were made in water with a ground-glass homogenizer, and assayed immediately. In the early stages of development tissues from about six eggs were pooled to make homogenate; in later stages, tissues from two egg were used GDH activity was stable in frozen homogenates 15 for 24 hr.

Attempts to release further activity from tissue notes Hogeboom & Schneider (1953) have shown of DH of mouse liver localized exclusively in the mitochondria these authors obtained a threefold increase of activity after disruption of the mitochondria When homogenates of liver were rapidly frozen and slowly thawed no increase in GDH activity occurred Acetone-dried powders prepared from hen liver by a method described showed less activity/mg of protein the genate

Enzyme concentration When different dilutions of the purified ox-liver GDH (Boehringer) were assayed a linear relationship was found between decrease in extinction and concentration of enzyme within the range 1·6 × 10⁻² units (1–16 μg of protein) When assayed within this range Olson & Anfinsen tained a maximal specific activity of 30 units per mg which is 300 times greater than that of the Boehringer preparation.

Reversibility of the reaction Olson & Anfinsen (1952) glutamate and ammonia was used as a substrate in

has been confirmed on homogenates of hen liver when DPN⁺-reduction values were one-fifth of those for DPNH oxidation. In view of the very low activities of GDH found in the tissues studied only the DPNH-oxidation reaction has been used for assay purposes.

Inhibitors. GDH is highly specific for L-glutamic acid (Euler, Adler, Günther & Das, 1938; Dewan, 1938; Olson & Anfinsen, 1953). The addition of L-glutamic acid (end to homogenates

concentration of substrate) to a homogenate of hen liver (0·9 mg. of protein) produced 60% inhibition of GDH activity. Vallee, Adelstein & Olson (1955) have shown that GDH contains 4–5 g atoms of Zn/mole of enzyme (mol.wt., 10⁶). They found that activity was inhibited significantly by the metal-chelating agents sodium diethyldithiocarbamate and 1:10-phenanthroline. GDH activity of the Boehringer preparation (100 μg.) was inhibited 50–80% by sodium

Answer: Yes; COULSON and JACOBS (*J. Chem. Soc.* 1949 1933) calculated by the method of molecular orbitals the bond orders in stilbene, 4-aminostilbene and 4:4'-diaminostilbene and the charge migrations in the last two compounds. The slide (Fig. 3) shows diagrammatically the charge distribution in the ground and first excited states in 4-aminostilbene. The radius of each black circle is proportional to the calculated excess π electron charge (above 1 0) on the atom. The change in bond order from ground to excited state is indeed greatest at the ethylenic double bond, as is also the case for stilbene itself, where it leads to the appearance of fine structure in the u.v. absorption spectrum due to the —C=C— vibrational frequency.

M. VAN LANGERMEERSCH: Concernant le déplacement des bandes d'absorption u.v. lorsqu'on change de solvant: l'effet constaté, lorsque les solvants sont de constitution chimique très différente, comme dans les examples cités, existe-t-il aussi lorsque les solvants font partie d'une série homologue (par exemple hexane et heptane)?

Answer: We have examined the spectra in other hydrocarbon solvents, such as *iso*-octane and liquid paraffin, and have commenced experiments to determine these effects in a homologous series of paraffin hydrocarbons and of alcohols. The effects noted in 13 different solvents appear to support the generalizations I have mentioned. These are the conclusions of the preliminary survey.

diethyldithiocarbamate (1 μmole) and αα'-dipyridyl (1 μmole). Significant inhibition of GDH activity was produced when either of these two compounds (0 1 μmole) was added to a homogenate of livers from 14-day-old chick embryos (corresponding to 0 48 mg. of protein).
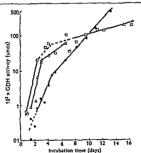
RESULTS

Egg yolk is known to contain many enzymes and it was not surprising to find GDH present in unincubated egg yolk of the fowl. Yolks obtained from eggs containing developing embryos showed a considerable increase of activity from the second day to 7·5 days of incubation (Table 1), and a decrease during the period 7·5–18 days of incubation. This behaviour is similar to that observed by Remotti (1927) for a protease having maximum activity in fowl-egg yolk at the tenth day of incubation, which is the time of maximum protein catabolism in the chick embryo (Needham, 1931). No GDH activity has been detected in egg white.

The total GDH activity of the chick embryo, the yolk sac and the vascular area of the yolk sac is

Table 1. *Changes of GDH activity in egg yolk during development of the chick embryo*

Incubation period (days)	$10^3 \times$ GDH activity	
	(Units/yolk)	(Units/ mg. of protein)
0	1130	0 32
1	1240	0 35
2	1140	0 63
7·5	4000	2·0
11	3600	1·0
18	600	0·47

Fig. 1. Levels of GDH activity in the chick embryo and the yolk sac during development. ●, Embryo; △, vascular area; □, yolk sac.

shown in Fig. 1, a semi-logarithmic plot being used for GDH activity. Activity was first detected in blastoderms, where it increased more rapidly than in the embryo until about the third day of incubation. This activity was not localized in the opaque area, as the vascular area showed the same rate of increase up to 3 days of incubation. After the third day of incubation GDH activity increased less rapidly until the yolk sac was entirely vascular (about the seventh day). The rate of increase of GDH activity in the total yolk sac in later stages was very much less rapid. GDH activity was first detected in the chick embryo at the 10 somites stage (35 hr. of incubation) and activity increased rapidly until the 4·5 days developmental stage, when a change in the rate of increase was observed. A similar change in growth rate (on a wet wt. basis) has been observed at this stage of development.

The distribution of GDH in the egg during development is shown in Fig. 2. GDH in the yolk is not severely depleted until the 16th day, when the embryo contains nearly half the total GDH in the egg. The amount present in the extra-embryonic tissues (the allantoic membrane is not shown as its contribution to the total is negligible), although greater than that of the embryo until the tenth day, is never more than 5 % of the total activity of the egg.

The GDH activities/g. wet wt. in the yolk sac and the vascular area of the yolk sac during 1–16 days of incubation are shown in Fig. 3. The activities/mg. of protein in the yolk sac follow a similar pattern to those relative to wet wt. except that the slight increase in activity/g. wet wt. at 4 days (Fig. 3) is accentuated on a protein basis (2 5 days, 0·6 × 10⁻³;
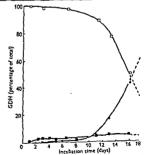

Fig. 2. Distribution of GDH in the hen's egg during incubation. ●, Embryo; ■, yolk sac; □, yolk.

The relative reactivities of deoxyribonucleotides and of
the bases of DNA towards alkylating agents

4 days, 1.4×10^{-3} units/mg. of protein) and no sharp rise in activity (on a protein basis) was found in the vascular area of the yolk sac just before the third day of incubation. However, at 6·5 days of incubation two high results were obtained for the vascular area (average 1.7×10^{-3} units/mg. of protein).

In the allantoic membrane, GDH activity/g. wet wt. showed only a very slight increase from 6 to 18 days of incubation (6 days, 16.5×10^{-3}; 11 days, 23×10^{-3}; 18 days, 24×10^{-3} units/g. wet weight). The increasing protein content of the allantoic membrane during this period of incubation showed as a decrease of activity/mg. of protein (6 days, 1.27×10^{-3}; 11 days, 0.51×10^{-3}; 18 days, 0.48×10^{-3} units/mg. of protein).

The opaque area of blastoderms possesses a higher activity than any other extra-embryonic tissue at any time (on a wet wt. and protein basis) during the whole period of development; there is a small peak on the fourth day of incubation which may be due to the sudden rise in GDH activity (on a wet wt. basis only) in the vascular area just before the third day.

The relative GDH activities (per g. wet wt. and per mg. of protein) in whole chick embryos during development (Fig. 4) again follow a similar pattern. GDH activity on a wet wt. basis increases slightly from the 10 somites stage to the second day of incubation, followed by a fall until the fifth day. Activity remains constant until the tenth day, after which there is a steady rise up to 16 days of incubation, when the embryo has reached the high level of the 2 days of incubation stage. On a protein basis, GDH activity exhibits a more marked peak on the second day of development, but otherwise activity remains nearly constant during later development. This peak of activity/mg. of protein is of the same order as that found in the opaque area of blastoderms 24 hr. earlier (2.6×10^{-3} units/mg. of protein).

From the fourth day the constant level of activity/mg. of protein in the embryo is five times greater than that of the yolk sac.

The increase of GDH activity (per g. wet wt.) in whole embryos after 10 days was further studied by measuring GDH in organs and tissues of the embryo. Tissues studied were liver (from 7 days), brain (from 10 days), heart and skeletal muscle (from 14 days until 3 days after hatching). The GDH activities/g. wet wt. of these tissues during late development of the embryo and early development of the chick (Fig. 5) remain constant in the brain and heart, but there is a slow increase in the liver up to the 16th day of incubation and then a decrease until 3 days after hatching, a similar pattern occurs in skeletal muscle, with a peak at 18 days of incubation. On a protein basis a slightly different accumulation of activity is evident (Fig. 6) the activities of heart and brain increase steadily after 19 days of incubation, liver shows a rather earlier peak (14 days) and
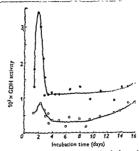

Fig. 4. Relative GDH activities in the developing embryo. ●, GDH activity/mg. of protein; ○, GDH activity/ 100 g. wet wt.
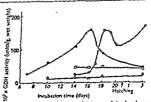

Fig. 3. Relative GDH activities in the yolk sac during development of the embryo. ▲, Vascular area of yolk sac; ■, yolk sac.

Fig. 5. GDH activity/g. wet wt. in tissues of the developing chick embryo. ●, Liver; ○, muscle; △, brain; □, heart.

TABLE I

REACTION OF DEOXYRIBONUCLEOTIDES ($0.1 M$ IN $0.4 N$ Na PHOSPHATE, pH 7.2)
WITH $0.1 M$ DIMETHYL SULPHATE FOR 1 h AT 37°C

| | Yield, % | | RF | | λ max. | ε290 mμ |
	(a)	(b)	(a)	(b)	mμ	ε260 mμ
Deoxyguanylic acid	—	60	—	0 65	282; 256	0.68
Deoxycytidylic acid	7	16	0.55	0.81	279	1.25
Deoxyadenylic acid	—	5; 14	—	0 65; 0.77	261; 260	0.13; 0 05
Thymidylic acid	19	—	—	0.38	—	—

TABLE II

ACTION OF NITROGEN MUSTARD (HN$_2$)
ON DNA

(0.01 moles P/l, WITH $0 01 M$ HN$_2$ IN
$0 036 N$ Na PHOSPHATE pH 7.2, FOR 24 h
AT 37°C)

	Control	Reacted
G/T	0 98	0 41
A/T	1.20	1 06
C/T	0 91	0 81
$\frac{A + T}{G + C}$	1.17	1.69

The question of whether the same order of re-
activity applies to these bases when present as
constituents of DNA was investigated by reacting
calf-thymus DNA with nitrogen mustard (HN$_2$,
di-(2-chloroethyl)-methylamine). Perchloric acid-
hydrolysates of treated and untreated DNA were
analysed by paper chromatography, according to
WYATT[4], with the addition that two-dimensional
chromatograms using isopropanol-NH$_3$[5] were run
for the former. Table II expresses the results in
terms of the molar ratios of the bases For the
control, recovery was 90 % moles base per mole P.
The same order of reactivity of the bases is evident,
and it can also be deduced that attack by alkylat-
ing agents may be expected to be greater on nucleic
acids containing a preponderance of guanine and
cytosine rather than of adenine and thymine, ac-
cording to the generally accepted classification of their analytical types[6].

From the reacted DNA three additional components were separated, having R_F values
with IPA/HCl and IPA/NH$_3$ resp. as follows.

0 02, 0 02, 0 05, 0.11; 0 20, 0.37

Of these, the second showed an ultraviolet absorption spectrum of the type expected for a

" ' .... ' The .... of the remaining components is being studied In view of the hypo-

.   ' .   ·   with mono-
                                                                   lecules, and
     ·   ' ·   ·   ·. ·   ay presents
some interest

I thank Professor J A V BUTLER and Mr E. W JOHNS for the supply of DNA.
The work has been supported by grants to the Chester Beatty Research Institute (Institute
                      '   .... ' '   .... from the British Empire Cancer Campaign the Jane
    ''   ''   ''
                                                                              J. D. LAWLEY

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1 A HADDOW, *Ann Rev Biochem*, 24 (1955) 706.
2 R MARKHAM AND J. D SMITH, *Biochem. J*, 49 (1951) 401.
3 P D. LAWLEY, *Proc Chem Soc*, (in the press).
4 G R WYATT, *Biochem J*, 48 (1951) 584
5 A D HERSHEY, J DIXON AND M CHASE, *J. Gen Physiol*, 36 (1953) 777.
6 E. CHARGAFF, in E CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. I. Chap 10, Aca-
    demic Press, Inc., New York, 1955.
7 P. D. LAWLEY AND C. A WALLICK, *Chem. & Ind. (London)*, (1957) 633
8 R J GOLDACRE, A LOVELESS AND W. C. J. Ross, *Nature*, 163 (1949) 667.

Table 2. *Comparison of GDH activities in organs from a 3-day-old chick and a 9-week-old hen*

		$10^3 \times$ GDH activity				
	(Units/g. wet wt.)			(Units/mg. of protein)		
	Chick (3 days)	Hen (9 weeks)	Increase (%)	Chick (3 days)	Hen (9 weeks)	Increase (%)
Heart	40	217	440	1 4	3 1	120
Liver	48	257	440	1·7	1·8	150
Brain	25	227	810	0·7	2·4	220
Muscle	173	515	200	1·8	10 3	470
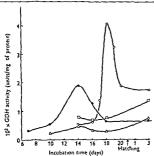
Fig. 6. GDH activity/mg. of protein in tissues of the developing chick embryo. ●, Liver; ○, muscle, △, brain; □, heart.

Table 3 *GDH activity per cell in early chick embryos*

Corrected incubation time (hr.)	$10^{-4} \times$ no of cells	$10^{12} \times$ GDH activity (units/cell)
34	1 0	240
36	1·2	220
47	4·3	93
53	8 2	61
56	12	53
60	15	53
70	92	15

skeletal muscle exhibits a nearly tenfold rise in activity from the 16th to the 18th day.

Leslie & Davidson (1951) have shown that there are large variations in cell mass and protein in the tissues of the chick embryo during development. The average cell number of a known weight of tissue was calculated from these authors' figures for deoxyribonucleic acid phosphorus ($2 35 \times 10^{-7} \mu g./$nucleus). The average GDH activity/cell in the developing tissues of the chick embryo presents a similar pattern to Fig. 6 except for muscle where GDH activity/cell continues to increase after the

18th day of incubation to 3 days after hatching The decrease in muscle-GDH activity on a wet wt. and protein basis after the 18th day of incubation (Figs. 5 and 6) now appears to be due to the great increase in muscle-cell mass and protein known to occur during this period of development (Leslie & Davidson, 1951).

For comparison with levels of GDH activity of embryonic organs, GDH activity has been measured in the corresponding tissues of a 9-week-old hen. The general increase in activity with age is shown in Table 2. The percentage increases in GDH activity on a wet wt. basis are in the ascending order muscle, heart and liver, brain; whereas the order on a protein basis is heart, liver, brain and muscle. The heart and liver show the same percentage increases in activity, the brain increases its activity/unit wet wt. by eightfold and activity/mg. of protein is increased nearly fivefold in muscle.

It is possible to calculate average GDH activity/cell in embryos during the 35–72 hr. incubation period, on the basis of the average number of nuclei/embryo, which has been found to show a steady exponential increase during this period (Solomon, 1957). The results (Table 3) show a gradually diminishing amount of enzyme/cell in the embryo despite the sharp peak of activity/mg. of protein at 2 days (Fig. 4). This may mean that many cells in the later stages of development contain little or no enzyme, i e. there may be a localization of enzyme activity.

The highest specific activity obtained for a preparation of ox-liver GDH is 1 unit/30 μg. of GDH preparation (Olson & Anfinsen, 1952) This figure may be used to calculate the maximum amount of GDH present in any tissue; the highest level of GDH activity/mg. of protein is 10 3 $\times 10^{-3}$ unit in skeletal muscle of the 9-week-old hen, which corresponds to 0 03% of the total protein. The maximum amount of GDH present in the 2-day-old chick embryo would be 0 01% of the total protein.

DISCUSSION

The increase of GDH activity in egg yolk from the second to the eighth day of incubation may be due to the disruption of yolk particles, e g. vitelline

platelets (Panijel, 1950), during the metabolism of the yolk. The comparatively large amount of GDH in the egg yolk during development is in excess of the requirements of the embryo until the pre-hatching stages; however, it is not yet known whether the GDH of the yolk is transferred to the embryo during later developmental stages or whether the embryo can synthesize GDH independently of the yolk.

The yolk sac is known to behave as a 'transitory liver' in respect to glycogen storage (Bernard, 1872) and as mature liver in its response to insulin (Zwilling, 1951). It is interesting to note that the high level of GDH activity/mg. of protein of blastoderms (2.6×10^{-3} unit) is greater than the activity of the yolk sac at 4 days (1.4×10^{-3} unit), vascular area at 6.5 days (1.7×10^{-3} unit) and the liver at 14 days (1.9×10^{-3} unit). The level of activity of 14-day-old chick embryo liver is identical with that of liver of the 9-week-old hen, and the liver at 14 days can be regarded as temporarily 'functionally mature' as far as GDH content is concerned. The high levels of GDH in the yolk sac during early development lend support to its suggested function as a 'transitory liver'.

The presence of minute amounts of GDH in tissues of the chick embryo does not indicate the extent to which the enzyme is being used for glutamic acid oxidation or synthesis. Both α-oxo-glutaric acid (Westfall, Peppers & Earle, 1955) and glutamic acid (Rupe & Farmer, 1955) are known to be present in 9-day-old chick embryos and their extra-embryonic tissue. Rupe & Farmer (1955) consider that the large reserves of free glutamic acid in the egg yolk and white are sufficient to satisfy the requirements of the developing embryo and, as

are in agreement with this suggestion, and may only act as a localized 'reserve mechanism' for ammonia fixation; however, Moog (1952) considers that chick embryonic tissues may not be provided with the enzyme reserves possessed by adult tissues.

When the daily increment of GDH accumulation relative to wet weight in the developing embryo is plotted, the curve shows some similarity with those of total carbohydrate and protein (Needham, 1931) in falling rapidly until the sixth day of development. After this time the GDH curve remains at a nearly constant level and resembles that of carbohydrate rather than protein, which rises rapidly after the seventh day of development.

Certain other enzymes which have been studied during the development of the chick embryo show some similarities to GDH. Levy & Palmer (1940) found that dipeptidase in the chick embryo had a high level of activity (on a wet weight basis) at

1.5 days of incubation, then activity decreased until the third day, and remained at a constant low level until the ninth day before rising to its former high level on the 16th day. This is a similar pattern to that of GDH after 2 days of incubation. The peak of GDH at 2 days precedes that of aminopeptidase (Levy & Palmer, 1943) at 2.5 days of development, which then follows a pattern similar to that of GDH during the later developmental stages. It appears that these peaks of activity in the early chick embryo follow the ontogenic sequence, dipeptidase, GDH and aminopeptidase, during 1.5-2.5 days of incubation. These peaks of activity in early embryos were not found for glutamotransferase and glutamine synthetase (Rudnick, Mela & Waelsch, 1953), which increase *pari passu* with total protein in the embryo and yolk sac (as did GDH after the fourth and sixth day respectively). Transferase activity (per mg. of protein) increased steadily in the liver between the sixth and 18th day of incubation but there was no peak of activity as with GDH. The change in GDH activity (per mg. of protein) in developing skeletal muscle shows a marked resemblance to that of adenosine triphosphatase (Moog, 1947), which increased threefold between the 12th and 20th days and then decreased to a constant level at 3 days after hatching. Moog (1952) states that the peak of adenosine triphosphatase activity coincides with muscular activity before hatching; the sharper increase (tenfold) in GDH activity in muscle during the 14th-18th day may also be associated with muscular activity of the embryo.
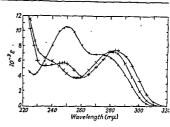
The levels of GDH (per mg. of protein) in tissues during development of the chick embryo are generally lower than in tissues of the 9-week-old hen. However, with GDH it has been shown that the opaque area of the blastoderm and the 2-day-old embryo have GDH activities as high as those of adult brain, heart and liver. The ratio of minimum to maximum activities in ascending order is chick embryo and allantoic membrane (3), heart (6), liver (7), brain (10), yolk sac (13) and muscle (26).

The GDH activity of early blastoderms is presumably localized in the opaque area since embryos of 10 somites have only traces of enzymic activity. The high level of activity (per mg. of protein) in the opaque area of the blastoderm rapidly falls with development and is followed by a surge of activity in the whole embryo at 2 days. It appears that the opaque area contains a high level of GDH during the formation of the primitive streak and early somites; a measurable amount of the enzyme occurs in the embryo after 24 hr., where it may have an important localized function in the morphogenic processes at this stage. The next surge of activity per mg. of protein occurs in the yolk sac at 4 days, but is not followed by a surge in total embryonic activity and may be connected with the mobilization of GDH in

acid, methylated at pH 7, yields 7-methylguanine on acid hydrolysis.[3] Further work, outlined here, has led to chromatographic isolation of a methylated deoxyguanylic acid, which yields 7-methylguanine on hydrolysis under much milder conditions than for the guanylic acid product, hydrolysis being appreciable at pH 7 and 37°.

Neutralized deoxy— . . . . . . hate (0·1M . . . . l.) in 0·4N . . . . 7·2, at 37°, for 1 hr. The mixture, chromatographed on Whatman No. 4 paper with saturated aqueous ammonium sulphate–propan-2-ol-0·1N-phosphate (79:2:19)[1] at pH 7·2, as solvent, afforded two components, one of R_r 0·4 (as for unchanged deoxyguanylic acid), and the other of R_r 0·65 and with absorption maxima at 282·5 and 256 mμ at pH 7. At 37°, in buffer of initial pH 7·14, the latter product changed slowly into another giving the absorption spectrum of 7-methylguanine, the half-life being ca. 20 hr. and the final pH 6·95. At 22°, a precipitate was gradually formed in the original reaction mixture which, unlike the original product, gave no reaction for deoxypentose.[5] This precipitate recrystallised from water as colourless needles (Found: C, 44·1, H, 4·3; N, 41·7. Calc. for $C_8H_7ON_5$: C, 43·6; H, 4·2; N, 42·4%), and its ultraviolet absorption spectra at various pH's were identical with those of 7-methylguanine (see Figure).[6] (These spectra show five isosbestic points, resembling those presented by Weissmann et al.[7] rather than those due to Gulland and Story,[8] in which only three isosbestic points occur although the extinction coefficients are of similar magnitude.) It appears therefore that, unless a rearrangement accompanies its hydrolysis, the initial product is 7-methyldeoxyguanosinium 5′-phosphate (I, or the isomer with the . . . . . . N . . . . 7-methyl- . . . . s deduced . . . . mμ) and 9800 (256 mμ), and hence that the yield was 60%.

A mechanism is therefore established by which alkylation of guanine moieties of deoxyribonucleic acid could cause, not only rather extensive changes in physicochemical characteristics due to quaternary

Product from hydrolysis of methylated deoxyguanylic acids:
×, pH 12; +, pH 7; ●, pH 1·3. Lines are for 7-methylguanine, at the same pH's

groups at ring-nitrogen atoms, but also elimination of the alkylated guanine moieties from the macromolecule, at physiological pH, by the slower subsequent hydrolysis of the 7-alkylguanine-deoxyriboside linkage. Further, the latter process might be expected to facilitate fission of the polymer chain, as suggested[9] for the analogous case of the apurinic acids.

The authentic sample of 7-methylguanine was a preparation by Dr. N. Anand, obtained through the courtesy of Dr. D. M. Brown and Professor F. Bergel, whom the author thanks. The helpful interest of Dr. E. M. F. Roe and Mr. G. M. Timmis is also much appreciated.

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the yolk. On the 14th day there is a pronounced surge in the liver which precedes a greater surge of activity in skeletal muscle on the 18th day. These surges of activity probably represent a pattern of functional differentiation of certain cells in the embryo, and in its liver and muscle.

SUMMARY

1. An increase of glutamic dehydrogenase (GDH) activity in yolk of the hen's egg has been shown to occur in the mid-incubation period.

2. Generally, the levels of GDH in embryonic tissues are less than those of tissues of the hen except for the blastoderm and 2-day-old embryo, which have GDH levels as high as those of some hen tissues.

3. GDH has been found to increase *pari passu* with total protein in the whole embryo and yolk sac from the fourth and sixth day of incubation respectively.

4. GDH activity in the chick embryo and its extra-embryonic tissues during development has been shown to have similarities with certain other enzyme activities.

5. In certain tissues of chick embryos during later developmental stages considerable surges of activity are found to occur which may be associated with functional differentiation.

REFERENCES

Bernard, C (1872). *C R. Acad. Sci , Paris*, 75, 55.
Boell, E. J. (1955). *Analysis of Development*, p 520. Philadelphia: W. B. Saunders Co. Ltd.

Braunstein, A. E. & Azarkh, R M. (1945). *J. biol. Chem*, 157, 421.
Braunstein, A. E. & Bychkov, S. M. (1939) *Nature, Lond.*, 144, 751.
Copenhaver, J. H., McShan, W. H. & Meyer, R. K. (1950). *J. biol. Chem.* 183, 73.
Dewan, J. G. (1938). *Biochem. J.* 32, 1378
Euler, H. von, Adler, E., Gunther, G. & Das, N. B. (1938). *Hoppe-Seyl. Z.* 254, 61.
Hamburger, V. & Hamilton, H. L. (1951). *J. Morph.* 88, 49.
Hogeboom, G. H. & Schneider, W. C. (1953). *J. biol. Chem.* 204, 233.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* 175, 385.
Leslie, I. & Davidson, J. N. (1951) *Biochim. biophys. Acta*, 7, 413.
Levy, M. & Palmer, A. H. (1940). *J. biol. Chem.* 136, 415.
Levy, M. & Palmer, A. H. (1943). *J. biol. Chem* 150, 271.
Meister, A. (1955). *Symposium on Amino Acid Metabolism*, p. 3. Baltimore: Johns Hopkins Press.
Moog, F. (1947). *J. exp. Zool.* 105, 209.
Moog, F. (1952). *Ann. N.Y. Acad. Sci.* 55, 57.
Morton, R. K. (1955). *Methods of Enzymology*, vol. 1, p 34. New York: Academic Press.
Needham, J. (1931). *Chemical Embryology.* Cambridge University Press.
Olson, J. A. & Anfinsen, C. B. (1952). *J. biol. Chem.* 197, 67.
Olson, J. A. & Anfinsen, C. B. (1953). *J. biol. Chem* 202, 841.
Panjel, J. (1950). *Biochim. biophys. Acta*, 5, 343
Remotti, E. (1927). *Ric. Morf.* 7, 199.
Rudnick, D, Mela, P. & Waelsch, H. (1953). *J. exp. Zool.* 126, 297.
Rupe, C. O. & Farmer, C. J. (1955). *J. biol. Chem* 213, 899.
Solomon, J. B. (1956). *Biochem. J.* 64, 15P.
Solomon, J. B. (1957). *Biochim. biophys. Acta*, 23, 24.
Sutherland, E. W., Cori, C. F., Haynes, R. & Olsen, N. S. (1949). *J. biol. Chem.* 180, 825.
Vallee, B. L., Adelstein, S J. & Olson, J. A. (1955). *J. Amer. chem. Soc.* 77, 5196.
Westfall, B. B., Peppers, E. V. & Earle, W. R. (1955). *J. nat. Cancer Inst.* 16, 337.
Zwilling, E. (1951). *Arch. Biochem. Biophys* 33, 228.

hypophysectomy may succeed. Incomplete extirpation of the organ accounts for a number of failures, especially in hypophyseal ablation by radioactive implants. Adaptation of the tumour to the altered 'endocrine environment' accounts for the ultimate failure so far of all these methods of treatment.

'Success' can be appreciated by the patient and measured by objective
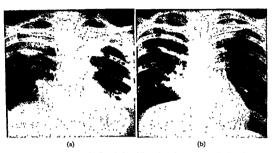

FIG. 1.—X-rays of chest. (a) Just before bilateral oöphorectomy and adrenalectomy. (b) A fortnight afterwards; to show the speed with which regression of metastases can occasionally take place (*Brit. med. J.*, 1956, ii, 627.)

methods. The relief of pain from bony metastases, for example, is often so rapid and dramatic that it is difficult to believe that it is due to tumour regression. It seems that some tension within the affected bone has been released and the pain thereby instantaneously dispersed. In other cases it may take weeks to disappear. Ulcers heal, superficial metastases and cutaneous recurrences regress and it is by measuring this shrinkage that a numerical assessment of improvement is possible. The colour photographs in Luft and Olivecrona's article of 1953 illustrate this point well. The reduction in size of a liver full of tumour is similarly available for measurement and indeed may be the most important facet of a case.

Occasional cases have been reported (Clain and Hunt, 1956) in which cerebral metastases have resolved and the patient has regained consciousness and returned to normal life. Pulmonary metastases sometimes disappear, pleural effusions may dry up and respiratory distress may be relieved (fig. 1). Pathological fractures often unite and paralysis due to spinal cord compression may completely recover.

All patients who have had endocrine surgery require cortisone, 25 to 50 mg. a day, for the rest of their lives. The dose must be increased during illness or following trauma, including surgical operations. Hypophysectomy has additional complications, such as myxœdema, which need appropriate

treatment. The temptation to give œstrogens in any form must be resisted because of the risk of stirring up the dormant tumour.

INDICATIONS FOR OPERATION

From reports therefore we know that a patient suffering from a metastasizing carcinoma of the breast has about a 50 per cent. chance of being relieved of her symptoms for a period varying between a few months and two or three years, if she undergoes one of these considerable operations. Everything should be done in the first place by local surgery and radiotherapy to postpone the day, because the relief obtained by endocrine surgery is limited in time. Androgens should always be given, particularly to the younger patients, and if the tumours respond to this treatment, the question of surgery should be further postponed. When the time for endocrine surgery finally arrives, the decision to operate usually depends upon a consideration of the type and site of the metastases and the degree of symptoms from which the patient is suffering. Other matters, however, which must also be considered concern the patient, her home conditions, her relatives, her desires, her outlook on life and her feelings about her disease.

It has been my experience that sometimes, when a patient has been recorded as having made a successful recovery following androgen therapy, another following bilateral oöphorectomy and adrenalectomy and possibly even a third following hypophysectomy, the situation in reality has been different. An impression that she has been snatched from the jaws of death on three separate occasions would be better expressed by saying that she has been permitted to suffer the agonies of dying three times over. This is not good treatment. It is an abuse of the powers that have been given us by the advance of modern scientific methods. We must only operate on those patients in whom the palliation may be judged worth while. If the patient is young, if prolongation of her life is genuinely desired by her husband or by her children, if her temporary presence is necessary to their future happiness or if the patient herself is intensely desirous of remaining with them and of being given a chance to lead a useful life for a little time longer, then, however ill she may be, endocrine surgery is justified, whatever the risks. It must be recognized therefore that it is not only the surgeon who can decide whether such surgery should be done. The general practitioner, the patient, her husband, her children or her other relatives should all be consulted or considered before this very serious treatment is undertaken. The patient's cooperation is an asset and in most cases it is better that she should be fully aware of the state of her disease. The operation gives her hope and any improvement is welcomed and appreciated with gratitude. If she does not know that her condition is due to cancer, improvement, however dramatic, will be no more than half appreciated, because too much will be expected and a grudge will be borne for not restoring normal health. This must not be taken as advocating that every patient should always be told the full truth. If they know it, so much the better.

INCREASE OF DEOXYRIBONUCLEIC ACID AND CELL NUMBER
DURING MORPHOGENESIS OF THE EARLY CHICK EMBRYO

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INTRODUCTION

While deoxyribonucleic acid (DNA) has been measured in chick embryos of later stages from eggs which had been incubated from 32 hours to 20 days, nothing is known about the rate of increase of DNA and the doubling rate of cells in the embryo during the important embryogenetic period immediately following the formation of the primitive streak. HOFF-JØRGENSEN[2] measured the DNA in incubated eggs of the domestic fowl by a microbiological method and found that the DNA content of the whole egg did not increase until after the third day of incubation This work gives no information the rapid rates of cell doubling and increase of DNA in embryos and their own bryonic tissues between the first and third day of incubation The increase in DNA tent of chick blastoderms growing *in ovo* has also been measured to serve as a control for experiments on DNA synthesis by isolated chick blastoderms grown *in vitro*

MATERIAL AND METHODS

Embryos and their extra-embryonic membranes were removed from the egg and the embryos identified morphologically by reference to the developmental stages of HAMILTON AND HAMBURGER[3]. The periods of incubation quoted by these authors for each stage were then used as "corrected in-

limb buds). In the stages of development before the ten-somite stage the blastoderm was removed from the

bryo was separated from its vascular area so that analyses could be carried out on the embryo and the vascular area separately This gave 3 fractions of tissue — "embryo with vascular area", "embryo", and "vascular area" (the area opaca in early stages) All tissues were stored at −20°C.
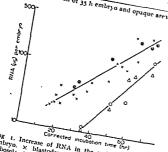
After weighing, the tissue fractions from two or more eggs were suspended in ice-cold 1.0% citric acid

stained nuclei determined by counting in a haemocytometer. For each determination the nuclei in 96 large squares were counted (usually about 1,000 nuclei) A nucleus undergoing mitosis was counted as one nucleus. The standard deviations ranged from 5–20%.

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blastoderms from 1.2–1.8 μg. The small number of determinations by the thymine method on embryos make it impossible to give an accurate figure

TABLE III

DNA CONTENT OF EMBRYOS DURING EMBRYOGENESIS

Embryos	Corrected incubation time (h)	No. embryos	Deoxyribose method μg DNA per embryo	Thymine method μg DNA per embryo	DNA (thymine method μg / ml)
	35	7			
	52	7	2.4 ± 0.2		
	70	15	7.7 ± 0.6	9.2 ± 0.2	
Blastoderms	19	4	45.0 – 1.5	61.5	
	35*	8	2.4 ± 0.2	3.1	
	37	7	9.2 ± 0.9		
	53	5	12.9 ± 0.9	13.0	
	56	5	14.8 – 1.0	13.0	
		5	24.4 ± 1.8	35.0	

* by addition of 35 h embryo and opaque area
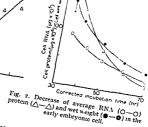
Fig. 1. Increase of RNA in the early chick embryo. × blastoderm (ribose); ⊗ blastoderm (uracil); △ embryo (ribose); ○ embryo (uracil).

Fig. 2. Decrease of average RNA (○—○) protein (△—△) and wet weight (●—●) in the early embryonic cell.

In blastoderms, the rate of increase of RNA is slightly slower than that of DNA, but that of protein, wet weight and DNA are very similar; however, in embryos there is an increase of DNA per wet weight (40 to 170 mg %) during the incubation period based on deoxyribose determinations, and from 93 to 240 mg % during the same period based on thymine determinations. NOVIKOFF AND POTTER[3] found about 110 mg % DNA in embryos after 2.5 days incubation. There is no variation in RNA per wet weight (500–600 mg %) in embryos up to 3 days of incubation; this level of RNA is again higher than the results obtained by NOVIKOFF AND POTTER[3] during the 2.5 to 4 day incubation period (350 mg %). These authors found a sharp decrease in RNA per wet weight after about 5 days of incubation.

The RNA/DNA ratio is greater in 24 h blastoderms (8) than in the embryo at 35 h of incubation (4). The ratio of RNA/DNA in the somites of the chick embryo

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2*

A method of tissue extraction for DNA similar to that of OGUR AND ROSEN[5] was used. About __ blastoderms, or groups of 2-8 embryos at later stages of development, were homogenized in

3 % of the amount in the nuclei was found (within the experimental error of the method).

The deoxyribose component of DNA was measured by reaction with indole using the method of CERIOTTI[6]. The volume of reagents used was one quarter of those used by CERIOTTI, so that DNA

RESULTS AND DISCUSSION

Nuclei counts of embryos with and without their vascular areas are plotted separately on a logarithmic scale against their corrected incubation times (Fig. 1). The exponential increase in cell number is then shown as a straight line. Results for the DNA content per embryo on a logarithmic scale show a similar trend but a much greater scatter (Fig. 2); if lines of the same slope as Fig. 1 are fitted to the results in Fig. 2, it is possible to demonstrate constancy of DNA per nucleus. In the embryos with their vascular areas,
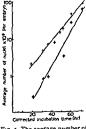
Fig. 1 The average number of nuclei per embryo during embryogenesis Embryo (O—O). Embryo and vascular area (●—●).
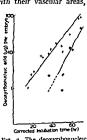
Fig. 2 The deoxyribonucleic acid content per embryo during embryogenesis Embryo (O—O); Embryo and vascular area (+—+).

Fig. 3. The average number of nuclei in the chick embryo (O—O), the vascular area (x—x), and yolk sac (△—△) during embryogenesis. ■

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during the 35-46 h incubation period is about 2.0 (calculated from the results of HERMANN[27]).

The rate of increase of RNA per embryo during the first three days of incubation is slower than that of the increase in the average number of nuclei per embryo[23]; this can alternatively be expressed as a decrease of RNA per cell. In Fig. 2 the decrease of RNA in the early embryonic cell is compared with the decrease in protein and wet weight. While the decrease of RNA and wet weight are similar, cell protein decreases more rapidly.

<div style="text-align:center">DISCUSSION</div>

Recent evidence for the occurrence of DNA in the hen's egg has been given by FRAENKEL-CONRAT et al.[28] who isolated a protein, avidin, from hen's egg white and found that it contained DNA. HOFF-JØRGENSEN[6] used a microbiological method for deoxyriboside (after depolymerisation of DNA with deoxyribonuclease) to determine DNA in the whole hen's egg and found that the unincubated egg contained 118 $\pm$ 12 μg DNA. This figure is somewhat lower than figures obtained in this work by the isotope dilution method with perchloric acid extraction (362 μg) and the phenol method (179 μg DNA). Although HOFF-JØRGENSEN[6] found no increase in DNA content of the incubated hen's egg until after the 3rd day of incubation, a considerable increase in cell population is known to occur in the egg during the first three days of incubation. At the third day of incubation, the chick embryo and its vascular area (excluding outer periphery of yolk sac) contain at least 80 μg DNA[24], and from HOFF-JØRGENSEN's results it might be concluded that nucleic acids in the egg yolk or white were being utilised by the developing embryo.

FRAENKEL-CONRAT et al.[28] using the method of SCHNEIDER[29] found 30-45 μg total nucleic acids per ml of egg white; this is roughly equivalent to 900-1350 μg nucleic acid per egg white. Average figures for total nucleic acid in egg white in this work are close to this range (828 μg by the perchloric acid method and 2068 μg by the phenol method). As egg white is a secretion of the oviduct it is not surprising to find relatively large quantities of RNA present and the DNA may be derived from cell debris in the oviduct[30].

HOTCHKISS[31] has suggested that this "cytoplasmic DNA" may be a precursor form and is not necessarily genetically specific material. It is possible that the DNA is present as a storage reserve[25, 31] to supply DNA for early embryological development until the embryo is capable of synthesising its own DNA. The nucleic acids (or similar highly polymerised compounds) may be a convenient means of storing nucleic acid precursors, which could be obtained by degradation when required by the embryo. The possibility remains, however, that the nucleic acids in the egg are largely non-functional in early embryogenesis and it is only during later development that these nucleic acids are degraded and used by the embryo in the same manner as other yolk and white constituents.

The content of acid-soluble pyrimidine and pyrimidine nucleotides of unincubated hen's yolk and white appears to bear some quantitative relation to the respective amounts of nucleic acid present. If these acid-soluble materials are about to serve as precursors for nucleic acid synthesis in the same way as suggested by MARRIAN[32] for purine precursors, the low amounts of acid-soluble pyrimidines might correspond to the slight requirement of the early blastoderm for such material.

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the average amount of DNA per nucleus from the two graphs is $1.0 \cdot 10^{-6}$ µg and remains constant throughout this period of development From individual determinations the average amount of DNA per nucleus is $1.1 \cdot 10^{-6} \pm 0.5 \cdot 10^{-6}$ µg. In the embryo, the average amount of DNA per nucleus from the graphs is only $6.2 \cdot 10^{-7}$ µg and from four individual results is $7.5 \cdot 10^{-7}$ µg; both these sets of figures are within the limits of error due to the methods and biological variation.

On a linear scale the average number of nuclei in the developing embryos follows an exponential curve (Fig. 3). However, the average number of nuclei in the vascular areas (obtained by difference between the two curves in Fig. 1, together with four individual results) also increases exponentially, as the area opaca becomes increasingly vascular, up to about 55 hour incubation time. After this time, the rate of increase of cell number of this mesodermal layer bounded by the sinus terminale decreases; then there is a rapid increase in the average number of cells of endoderm (calculated from DNA results) which is spreading round the yolk to form the yolk sac

As the number of cells has been determined in embryonic and extra-embryonic tissues during the 20–55 hour incubation period, it is interesting to compare the variation in the rate of doubling of cells from fertilisation using the data of OLSEN[6] for the cleavage stages (one to 256 cells). The instantaneous rate of increase in cell number (k) is calculated according to the formula of BRODY[8] and the doubling time (t_2) is then obtained from $t_2 = \log_e 2/k$. After the first cleavage, which according to OLSEN takes place about 5 hours after fertilisation, the doubling rates are between 0 8 and 1 0 hour up to the 256-cell stage. The average time of doubling from 256 cells to 2 10⁶ cells (primitive streak stage) is 2.3 hours and this increases to 8.9 hours during embryogenesis (Fig. 4). The average doubling rate for cells of the embryo, without its extra-embryonic membranes, from the definitive streak stage to soon after limb bud formation (70 hour incubation) is 6.2 hours.

The DNA content per wet weight of the embryo increases during the 36–70 hour incubation period from 40 to 175 µg DNA per 100 mg wet weight; this means that in the early chick embryo the wet weight per cell is decreasing during embryogenesis (Fig. 5). In the vascular area
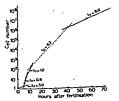
Fig 4 Comparison of doubling rates of cells during cleavage of the hen's egg and early morphogenesis of the chick embryo t_2 = rate of doubling in an hour.

Fig. 5. The decrease of protein (△—△) and wet weight (O—O) per cell during early morphogenesis of the chick embryo.

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In hen's egg white, there is over ten times more RNA than DNA (by the phenol method); but in the yolk there is only three times more RNA than DNA When the results obtained by perchloric acid extraction are compared, no such differences in RNA and DNA content of the hen's egg are observed

The RNA/DNA ratios of the 10 somite embryo (4) and blastoderm (8) are somewhat higher than the ratios found in most tissues of the chick embryo during later developmental stages[27, 33, 34]. This is in accord with the findings of CASPERSSON[35] and BRACHET[36, 37] that nucleic acids are abundant in cells which are rapidly synthesising protein.

Previously reported values for amounts of DNA per nucleus for fowl tissues and erythrocytes are within the range $2.1-2.6 \cdot 10^{-6}$ μg[13, 38, 39] NOWINSKI AND YLSHOK[10] using the Dische reaction, obtained values as high as $8.2 \cdot 10^{-6}$ μg DNA per nucleus in the wings and legs of chick embryos. KURNICK[41] measured DNA by reaction with methyl green in fowl erythrocytes and reported low values of DNA per nucleus ($1.6-1.8 \cdot 10^{-6}$ μg). Results obtained using the thymine method for DNA show a wide scatter between $1.0 \cdot 10^{-6}$ and $2.1 \cdot 10^{-6}$ μg DNA per nucleus in embryos but these for blastoderms (Table III) are in reasonable agreement with those obtained by KURNICK[41].

The rapid rate of cell division during embryogenesis of the chick may be the dominant factor in its metabolism The rates of increase of wet weight and protein are not sufficient to keep up with cell division and DNA synthesis, consequently there is an apparent loss of wet weight and protein per embryonic cell This state only exists for the first three or four days of incubation after which the instant the rate of increase of DNA becomes less (from results of NOVIKOFF AND POTTER) and the cell protein and wet weight slowly increase during later stages of differentiation[33, 42].

The various opinions on the relationship between cell division and differentiation in embryonic development have been admirably summarised by HUGHES[43] It is probable that the metabolism of the early chick embryo is geared to maintain a high rate of cell division even at the expense of some of the cell constituents. During this period, the fundamental embryonic structures are formed and it is only when cell division slows down that the embryonic cell is able to elaborate its metabolism to promote functional differentiation.

the ratio DNA/wet weight remains constant during the 30 to 70 hour incubation period.

Measurements of the protein content of embryos by the tyrosine method (using egg albumen as standard protein) showed the same rate of increase in protein for this period of development as those for protein nitrogen[9]; the protein per cell during this period showed a steady decrease until after 60 hour incubation time (Fig. 5). LEVY AND PALMER[10] found that there was a decrease of protein nitrogen per wet weight during the first four days of development of the chick embryo; the same relationship is seen in Fig. 5 The DNA[1] and protein nitrogen[9] results for embryos after the period of development studied in this work indicate that the protein per cell decreases less rapidly after three days, and after five days incubation time the protein per cell remains constant and then increases during later development. So that a characteristic of embryogenesis appears to be a very rapid increase in cell number accompanied by a loss of cell protein and to a lesser extent of cell wet weight. After embryogenesis, the rate of cell division gradually decreases and the steady rate of increase of protein nitrogen per embryo eventually becomes an increase of protein per cell; this latter process is the expected characteristic of later differentiation processes of embryonic cells[11].

The DNA required for the rapid rate of cell division in early chick embryos may be obtained by direct incorporation of DNA from the egg yolk or albumen. This would explain the unchanged DNA content (118 ± 12 µg) of the whole egg during the first three days incubation as found by HOFF-JØRGENSEN[2]. Measurements of the average DNA content of egg yolk and albumen during the period from laying to 72 hour incubation with the corrected Ceriotti method showed that there is about 400 µg DNA in the yolk and 460 µg in the albumen of each egg. Some of this DNA may be utilized by the embryos during early embryogenesis, but early chick embryos may also be capable of synthesising their own supply of DNA for their rapid cell division; this latter process is now being investigated by experiments *in vitro*.

SUMMARY

The rates of cell doubling and increase of deoxyribonucleic acid in chick embryos and their extra-embryonic tissues have been studied between the first and third day of incubation. During this period embryogenesis appears to be characterized by a rapid increase in cell number accompanied by a loss of cell protein and, to a lesser extent, of cell wet weight

REFERENCES

[1] A. B. NOVIKOFF AND V. R. POTTER, *J. Biol. Chem*, 173 (1948) 233
[2] E. HOFF-JÖRGENSEN, *Recent Develop. Cell Physiol*, Proc. 7th Symposium Colston Research Soc. Univ. Bristol. 1954. P 79.
[3] V. HAMBURGER AND H. L. HAMILTON, *J. Morphol.*, 88 (1951) 49.
[4] L. C. SZE, *J Exptl Zool.*, 122 (1953) 577.
[5] M OGUR AND G ROSEN, *Arch. Biochim. Biophys.*, 25 (1950) 262.
[6] G CERIOTTI, *J Biol. Chem*. 198 (1952) 297
[7] M. W OLSEN, *J Morphol.* 70 (1942) 513.

. . . . ibridge, 1931, p. 921.

SUMMARY

The nucleic acid content of hen's egg white and yolk has been measured by isotope dilution methods using perchloric acid extraction and phenol partition techniques. The origin and possible physiological function of the nucleic acids is discussed.

Nucleic acids have been measured by colorimetric and isotope dilution methods in blasto-derms and chick embryos up to the third day of incubation. A dominant factor in embryogenesis of the chick appears to be a high rate of cell division and accumulation of DNA, which is greater than that of RNA and protein accumulation and increase of wet weight

REFERENCES

1

2

3

4

5 H HERMANN, M J. B. SCHNEIDER, B. J NEUKOM AND J. A. MOORE, *J. Exptl. Zool* , 118 (1951) 243

6 E HOFF-JÖRGENSEN, *Recent Develop. Cell. Physiol* , Proc. 7th Symposium Colston Research Soc. *Univ. Bristol*, 1954, p 79

7 J B SOLOMON, *Biochim Biophys Acta*, (in the press)

8 K. S. KIRBY, *Biochem J.*, 56 (1956) 405.

9 K S. KIRBY, *ibid* , (in the press)

10 V. HAMBURGER AND H L. HAMILTON, *J. Morphol* , 88 (1951) 49

11 M OGUR AND G ROSEN, *Arch Biochem. Biophys.*, 25 (1950) 262.

12 A. MARSHAK AND C MARSHAK, *Exptl Cell Research*, 5 (1953) 288

13 M C. DURAND, *Compt rend* , 241 (1955) 1340.

14 A MARSHAK AND H. J VOGEL, *J. Biol Chem.*, 189 (1951) 597.

15 G R WYATT, *Biochem J* , 48 (1951) 584.

16 J R FRESCO AND R C WARNER, *J. Biol. Chem.*, 215 (1955) 751.

17 E. R HOLIDAY AND E A JOHNSON, *Nature*, 163 (1949) 216.

18 E VOLKIN AND W E COHN, in D. GLICK, *Methods of Biochemical Analysis*, Vol 1, Interscience, Publishers, Inc , New York, 1954, p. 287.

19 J M. PLOESER A

20 D SHUGAR AND

21 D RAPPORT, A   1950) 421.

22 G CERIOTTI, *J Biol Chem* , 214 (1955) 59

23 G CERIOTTI, *ibid* , 198 (1952) 297

24 J B SOLOMON, *Biochim Biophys Acta*, 23 (1957) 24.

25 J B SOLOMON, *ibid* , 23 (1957) 211

26 E W. SUTHERLAND, C F CORI, R. HAYNES AND N. S OLSEN, *J. Biol. Chem.*, 180 (1949) 825.

27 H HERMANN, *Ann N Y Acad. Sci.*, 55 (1952) 99.

28 H FRAENKEL-CONRAT, N S SNELL AND E D DUCAY, *Arch. Biochem. Biophys* , 39 (1952) 80

29 W C SCHNEIDER, *J. Biol Chem* , 161 (1945) 293.

30 R JEENER, private communication

31 R D HOTCHKISS, in E CHARGAFF AND J N. DAVIDSON, *The Nucleic Acids*, Vol. 2, Academic Press Inc , New York, 1955, p 435.

32 D H MARRIAN, *Biochim Biophys. Acta*, 14 (1954) 502

33 J LESLIE AND J N DAVIDSON, *Biochim Biophys. Acta*, 7 (1951) 413.

34 R BIETH, P MANDEL AND R STOLL, *Comp. rend. soc. biol* , 142 (1948) 1020.

35 T CASPERSSON, *Naturwiss.*, 29 (1941) 33

36 J BRACHET, *Arch. biol (Liège)*, 53 (1942) 207

                                    Quant. Biol , 12 (1947) 18.
                                    949) 666.
                                    LLIE AND R Y. THOMSON, *Biochem J.*, 46 (1951) xl.
                                    ochim Biophys Acta, 11 (1953) 497.
                                    950) 151.

42 P MANDEL, R BIETH AND R STOLL, *Bull soc. chim biol* , 31 (1949) 1335

43 A. HUGHES, *The Mitotic Cycle*, Butterworths, Scientific Publications, London, 1952, p. 167.

Nucleic acid content of the egg of the domestic fowl

Early work on nucleic acids in unincubated eggs has been discussed by BRACHET[1] and recent literature on deoxyribonucleic acid (DNA) in eggs has been reviewed by HOTCHKISS[2]. Recently DNA has been measured in the whole hen's egg[3] and FRAENKEL-CONRAT et al[4] isolated a ribonuclein, a protein of hen's egg white and found that it contained DNA. Further quantitative evidence for the presence of both ribonucleic acid (RNA) and DNA in egg white and yolk using an isotope dilution technique is reported here.

Unincubated egg yolk and white (from Rhode Island Red hens) from 6–10 eggs were separately homogenized in water with a Waring blendor. Free nucleotides were removed from these homogenates by precipitation of protein with perchloric acid (0.2 M at 0°C), and after removal of lipid with ethanol and ether, nucleic acids were extracted with 1.0 M perchloric acid at 70°C for 1 hour. The method of KIRBY[5,6] for obtaining nucleic acid extracts was also used. Sodium ... ... or yolk and the mixture
for 1 hour After centrifu
for 2 days at 0°C (after
s were removed and the

RNA in these extracts was measured by an isotope dilution method for uracil[7], and DNA by the same method for thymine[8]. RNA was also determined by the orcinol method[9] after removal of free hexoses[10], and DNA by reaction of indole with the deoxyribose component[11] with a correction for absorption at 520 mμ[12]; neither of these colorimetric methods produced reliable results in extracts of egg yolk or white. Previously reported[13] amounts of DNA in egg yolk and white based on the latter colour reaction are probably erroneous owing to colour contamination ...

F   De at
t   for tr
n   er r e.
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pyrimidine was then purified by running the whole digest on at least four successive paper chromatograms[6]. By comparison of the initial and final specific activities (counts per minute) of the pyrimidine, the amount of pyrimidine present in the extract was determined. Results are based on nucleic acid preparations obtained from rat liver by the method of KIRBY[5,6]. The RNA content of the RNA was 6.3%, and the thymine content of the DNA was 10.5%. Counting and ultraviolet absorption errors were experimentally determined and are expressed as coefficients of variation in Tables I and II.

<table>
<tr><th colspan="3">TABLE I</th></tr>
</table>

"ACID-SOLUBLE" URACIL AND THYMINE DERIVATIVES IN EGG WHITE AND YOLK		
	Uracil (µg per white or yolk)	Thymine (µg per white or yolk)
White	460 ± 55	19 ± 4
Yolk	13 ± 1	6.6 ± 0.3

<table>
<tr><th colspan="3">TABLE II</th></tr>
</table>

NUCLEIC ACIDS IN EGG WHITE AND YOLK		
	RNA (µg per white or yolk)	DNA (µg per white or yolk)
Yolk		
Perchloric acid extraction	68 ± 14	52 ± 6
		57 ± 7
Phenol partition	114 ± 14	39 ± 2
White		
Perchloric acid extraction	520 ± 33	308 ± 16
Phenol partition	2260 ± 100	137 ± 30
	1595 ± 50	142 ± 5

The amounts of "acid-soluble" uracil and thymine derivatives are given in Table I; as the acid-soluble extract was digested with 70% (w/v) perchloric acid, these figures represent total free pyrimidine and pyrimidine nucleotide material. The nucleic acid content of egg white and yolk is given in Table II. The RNA content of the white is very much greater than that of the yolk, and the phenol partition method appears to extract more RNA than the perchloric acid method. Perchloric acid appears to give a better extraction of DNA than the phenol method; ... ... washing of the phenol layer. The ratio of amounts of ...ied by the phenol method) to amounts of the respective is approximately constant (6.5).

SYNTHESIS OF NUCLEIC ACIDS BY CHICK BLASTODERMS
GROWN ON SYNTHETIC MEDIUM

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HOFF-JØRGENSEN[1] found that there is a constant level of deoxyribonucleic acid (DNA)
in the whole hen's egg during the first three days of incubation. However, during
this period considerable cell division and morphogenesis of the rapidly growing
blastoderm occur, and this is accompanied by a rapid rate of increase of ribonucleic
acid (RNA) and DNA[2]. The hen's egg yolk and white has recently been found to
contain relatively large amounts of DNA[3], compared with the requirements of the
early embryo. HOFF-JØRGENSEN's results may thus indicate that the developing
blastoderm is obtaining DNA from the egg white and yolk. However, it is also possible
that the chick blastoderm can synthesise its own supply of DNA during embryo-
genesis. SPRATT[4,5] has shown that the explanted chick blastoderm will undergo
morphogenesis when grown on synthetic medium containing glucose as the sole carbon
source. This technique has been used in this work to demonstrate that the explanted
blastoderm is capable of synthesising RNA and DNA during embryogenesis.

METHODS

The technique for growing blastoderms *in vitro* is the same as that used by SPRATT[4,6]. The culture
medium consisted of 34 ml chick Ringer solution (sodium chloride 0.9 g, potassium chloride . . . . . .

. . . .

Ringer, glucose, phenol red solution and agar were pooled, and after neutralisation with sodium
bicarbonate, were autoclaved with the phosphate buffer solution. The phosphate and bicarbonate
buffer solutions were then added and the medium pipetted (2 ml portions) into watch glasses,
which were placed in Petri dishes containing sterile pads of moistened cotton wool and allowed
to cool to 38° C. All glass apparatus was sterilised in the autoclave for 20 min and dissecting
instruments were dry-sterilised for 4 h at 110° C . . . . 20–36 hours.
. . . . moved and the
. . . . Blastoderms
. . . . BÜRGER[7]. The
. . . . 18–26 hours.
. . . . according to
. . . .
chloric acid method previously described[3]. The average number of . . . .

* Present address.

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As egg white is a secretion of the oviduct, it is not surprising to find large quantities of RNA present and the DNA may be derived from cell debris in the oviduct. The amount of "cytoplasmic DNA" in the yolk represents a great excess over nuclear DNA in the early blastoderm as even after 24 h incubation a blastoderm contains only about 3 µg DNA[12].

It is possible that this "cytoplasmic DNA" and RNA (or similar highly polymeric compounds) are a convenient means of forming a storage reserve of nucleic acid precursors which could be obtained by degradation when required by the embryo.

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[1] J. BRACHET, *Embryologie Chimique*, Desoer, Liège, 1945.
[2] R D HOTCHKISS, in E CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Academic Press Inc., New York, Vol 2, 1955. p. 435
[3] E. HOFF-JORGENSEN, *Recent Develop. Cell Physiol*, Proc. 7th Symposium Colston Research Soc. Univ. Bristol, 1954, p 79
[4] H FRAENKEL-CONRAT, N S SNELL AND E. D. DUCAY, *Arch Biochem. Biophys*, 39 (1952) 80.
[5] ⬚ ⬚, ⬚ ⬚, ⬚ 56 (1956) 495
[6] ⬚ ⬚ ⬚, ⬚ 5 (1953) 288
[9] G CERIOTTI, *J. Biol Chem.*, 214 (1955) 59
[10] D RAPPORT, A. CANZANELLI AND R. GUILO, *Am J. Physiol.*, 162 (1950) 421.
[11] G CERIOTTI, *J Biol. Chem*, 198 (1952) 297
[12] J B SOLOMON, *Biochim. Biophys. Acta*, 23 (1957) 24
[13] A. MARSHAK AND H J. VOGEL, *J Biol Chem*, 189 (1951) 597
[14] W C SCHNEIDER, *ibid.*, 161 (1945) 293

* Present address.

was determined by
measured by the or
alkali[10], and by an i
with deoxyribose[11] r
were modified so th:
determinations in th . . . . (10 μg RNA = 1 μg DNA). Protein was determined by
the method of SUTHERLAND et al.[12].

RESULTS AND DISCUSSION

Five groups of blastoderms (batches 1–5), in which the notochord was just visible
(HAMILTON AND HAMBURGER's stage 5), were explanted on glucose medium and
incubated at 38° C for 18 or 26 hours. The increase in amounts of RNA, DNA and
protein per blastoderm during this period are shown in Table I. It can be seen that
there is no correlation between the time of incubation *in vitro* and the extent of
morphogenesis (as shown by the average number of somite pairs in each batch) and
morphogenesis is usually slower than normal (*cf.* WADDINGTON[13] and SPRATT[8]). The
amounts of RNA, DNA and protein in the blastoderm after incubation *in vitro* are
compared with amounts previously found in blastoderms grown *in ovo* after the same
incubation period. The amounts of DNA in blastoderms grown *in vitro* are generally

TABLE I

NUCLEIC ACID SYNTHESIS BY 20-HOUR BLASTODERMS GROWN ON GLUCOSE MEDIA

Batch No.	1	2	3	4	5
No. of blastoderms	2	4	7	4	3
Duration of incubation time *in vitro* (h)	26	26	26	26	18
Average no. of somite pairs after growth *in vitro*	6	7	10	11	13
Average no. of somite pairs after growth *in ovo* (controls)	16	16	16	16	11
DNA					
μg per blastoderm grown *in vitro* (by deoxyribose)	10.6	8.7	9.4	7.7	5 0
(by thymine)	—	—	8 8	—	—
μg per blastoderm grown *in ovo* (controls)	17.6	18.7	17.6	18.7	9 0
% controls	60	47	53	41	56
μg synthesised per blastoderm	7 5	5.3	6.0	4.3	3.2
Average no. of cells per blastoderm × 10⁶	—	—	—	—	6 4 (7,2)*
RNA					
μg per blastoderm grown *in vitro* (by ribose)	117	75	81	60	42
(by uracil)	107	71	—	—	—
μg per blastoderm grown *in ovo* (controls)	102	108	102	108	62
% control	110	68	79	56	68
μg synthesised per blastoderm	84	46	53	31	17
Protein					
μg per blastoderm grown *in vitro* × 10²	5.2	4 7	8.7	4.9	6 6
μg per blastoderm grown *in ovo* (controls) × 10²	24	26	24	26	11
% control	22	18	36	19	60

* control *in ovo*

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NUCLEIC ACID CONTENT OF EARLY CHICK EMBRYOS
AND THE HEN'S EGG

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Early literature on nucleic acids in chick embryos and the hen's egg has been reviewed by NEEDHAM[1] AND BRACHET[2]. The nucleic acid content of developing chick embryos (from the second to twentieth day of incubation) has been measured by NEEDHAM AND POTTER[3]; REDDY, LOMBARDO AND CERECEDO[4] made similar measurements in the latter half of this period of development. HERMANN, SCHNEIDER, NEUKOM AND MOORE[5] have measured the increase in nucleic acid content of the developing embryos of early chick embryos and found that protein nitrogen and nucleic acids accumulate at a similar rate, but that the increase in wet weight was more rapid HOLT-JØRGENSEN[6] determined the amount of deoxyribonucleic acid (DNA) in the whole hen's egg and found that no increase of DNA occurred until after 3 days incubation of the egg This constant level of DNA in the whole egg during the first three days of development is remarkable since embryogenesis and considerable cell division are known to occur in the egg during this period.

The nucleic acid content of the unincubated hen's egg, blastoderm and embryos during the first three days of incubation has been measured in this work by conventional colorimetric methods and by highly specific isotope dilution methods for uracil (RNA) and thymine (DNA). The growth curves obtained serve as a control for experiments on nucleic acid synthesis by isolated blastoderms[7] and reveal certain characteristics of the average embryonic cell during embryogenesis

MATERIALS AND METHODS

Ribonucleic acid (RNA) and DNA were prepared from livers of the rat (August strain) by the methods of KIRBY[8],[9]. Uracil-2-[14]C (Californian Research Foundation, specific activity 1 mc/mmole) and thymine-2-[14]C (Isotope Specialities Co, specific activity 0 3 mc/mmole, Californian Research Foundation, specific activity 1 mc/mmole) were used in the isotope dilution methods.

Unincubated eggs (6–10) were divided into egg white and yolk fractions, others were incubated at 37° for varying periods up to 3 days Embryos and extra-embryonic tissues were separated from the egg yolk, placed in ice-cold isotonic saline and their morphological development classified according to the development stages proposed by HAMBURGER AND HAMILTON[10] The average incubation time quoted by these authors for each stage was used so that morphological development is expressed as "corrected incubation time" Blastoderms were removed *whole until the 10-somite stage*; in later stages of development, the opaque area was dissected around the periphery of the vascular area (sinus terminale) and then the embryo was removed by dissection of the pellucid area The three *tissue fractions thus obtained were respectively* "blastoderm", "vascular area" and "embryo". All tissues were stored at ~20° C.

lower than those grown *in ovo* (41–60%) but there is a definite synthesis of DNA
in vitro as shown by the increased amounts of DNA per blastoderm (3.2–7.5 μg) a
those of the explanted blastoderm[2]. Blastoderms (20 h) growing on glucose med....
synthesise comparatively large amounts of RNA (17–84 μg per blastoderm) ...
are generally slightly less than the amounts produced by blastoderms grow...
(56–110% of normal). However, the amounts of protein synthesised appe...
small (18–60% of normal), which implies that a redistribution of protein with...
blastoderm is taking place so that embryogenesis may proceed.

Nucleic acid synthesis in later stages of embryogenesis was studied by gr...
blastoderms with 5–10 somite pairs on glucose medium for 20 h (Table II ...
this period of incubation, morphogenesis proceeded nearly as rapidly as...
(except for batch 6). As would be expected, the amounts of DNA synthesis...
(6.9–18 μg per blastoderm) are greater than when starting with 20-hour b...
and the amounts per blastoderm are correspondingly higher (48–122% of...
However, the amounts of RNA synthesised are lower (except for batch 5) than...
the earlier stages of embryogenesis.

TABLE II

NUCLEIC ACID SYNTHESIS BY 5–10 SOMITE BLASTODERMS GROWN ON GLUCOSE MEDIA FOR 20 HOURS

Batch No.	6	7	8	9	10
No of blastoderms	3	2	2	5	1
Average no of somite pairs after growth *in vitro*	13	19	19	20	22
Average no. of somite pairs after growth *in ovo* (controls)	26	30	17	17	30
DNA					
μg per blastoderm *in vitro* (by deoxyribose)	15.3	23	23	16 7	15 4
(by thymine)	—	—	—	21 3	—
μg per blastoderm grown *in ovo* (controls)	26	32	18 8	18.8	32
% control	59	72	122	101	48
μg synthesised per blastoderm	8 3	14 5	18	14	6 9
Average no. of cells per blastoderm × 10⁶	—	—	—	13 (15)*	12 4 (26 0)*
RNA					
μg per blastoderm *in vitro* (by ribose)	82	—	164	42	70
(by uracil)	—	—	—	65	55
μg per blastoderm grown *in ovo* (controls)	140	—	109	109	163
% control	58	—	150	49	39
μg synthesised per blastoderm	32	—	124	14	12

* control *in ovo*

In three batches, the average number of nuclei per blastoderm was determined
and in each case was found to be below normal. The average amounts of DNA per
nucleus were 0.8·10⁻⁴, 1.3·10⁻⁴ and 1 2·10⁻⁴ μg in batches 5, 9 and 10, respectively,
thus is near the range found for blastoderms growing *in ovo* (1.2·10⁻⁴ – 1.8·10⁻⁴ μg
DNA per nucleus).

The RNA/DNA ratios of blastoderms grown *in vitro* can be compared with values

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in di:

ROSEN[11] was used. All preliminary extractions were made at 4° C. The homogenate was brought to 0.2 M perchloric acid and stirred for 15 min; the suspension was centrifuged and the deposit extracted again for 5 min with 0.2 M perchloric acid. This extract is termed "acid-soluble fraction" and only such extracts from egg yolk and white were retained. Lipid material was removed from the deposit obtained after centrifugation by similar extraction with ethanol (twice), three ethanol-ether (1:2) extractions and the solid was then dried. In early experiments, the RNA was obtained from this powder by extraction overnight with 1.0 M perchloric acid (1 ml/0.5 mg powder) followed by two similar extractions for 15 min. However this procedure usually failed to remove all of the RNA, which then appeared in the DNA fraction (three extractions with 1.0 M perchloric acid at 70° for 20 min). Accordingly, RNA determinations were made on the pooled RNA and DNA fractions and, in the case of egg white and yolk, the RNA extraction step was eliminated and RNA and DNA determinations made on the final hot perchloric acid extract

KIRBY[8] has extracted RNA from various mammalian tissues by removing protein and DNA with phenol. This method has been modified (KIRBY[9]) so that RNA and DNA remain in the aqueous layer, and has been used in this work to extract RNA and DNA from egg yolk and white. The homogenate of egg yolk (or white) was made to 6% (w/v) with respect to sodium p-aminosalicylate, which was dissolved by stirring for 15 min at 4° C. An equal volume of 90% (w/v) aqueous phenol solutions was then added and the mixture stirred vigorously for 1 h at room temperature. The mixture obtained from egg yolk was centrifuged on a Spinco centrifuge at 20,000 r.p.m. for 1 h at 0° C, when it separated into three layers. The lowest layer consisted of phenol and protein, (there was an interphase of insoluble protein between the phenol and aqueous layers), then an aqueous layer containing RNA and DNA, and a yellow upper layer containing lipid. The lipid and aqueous layers were removed by suction and the residue extracted with an equal volume of water and again centrifuged. The combined aqueous and lipid layers were then extracted three times with ether to remove lipid and the residual ether removed by blowing nitrogen through the aqueous layer. When this procedure was applied to egg white, centrifugation at 2,000 r p m for 1 h on an International centrifuge was sufficient to separate the phenol and aqueous layers and no ether extraction was necessary. The extracts were then dialysed against distilled water (20 vols) to remove sodium p-aminosalicylate and nucleotides of low molecular weight. After three changes of water (during 2 days) the contents of the dialysis bag were colourless.

Nucleic acids were measured by estimation of the uracil content of RNA extracts and the
. . . . nucleotides
. . . . s of nucleic
. . . . l to dryness
under reduced pressure and suspended in 70% perchloric acid (0.5 ml). The nucleic acids were
. . . . treatment has been shown to give a maxi-
. . . . ytosine to uracil[16]. A known amount of
. . . . which was shaken thoroughly and allowed
to cool for 1 h. The perchloric acid was then precipitated as its potassium salt (the solution was kept slightly acid) and the insoluble salt spun down. The supernatant solution was concentrated under reduced pressure to 0 5–1.0 ml for application to a paper chromatogram. This precipitation

The whole supernatant from the digest was applied in a band (2–3 cm) on a paper strip of Whatman No. 1 paper (4 cm wide at the bottom and tapering to 2 cm wide after 10 cm). The chromatogram was developed overnight using n-butanol–0.1 N NH₃ (7:1)[14]; after the papers were dry, the bases were detected by ultraviolet absorption[17] and the radioactive pyrimidine was located by scanning the chromatogram for radioactivity. The radioactive area of the chromatogram was cut out and eluted by applying drops of 0.1 N HCl to the top of the rectangle of paper which was inserted in the top of a small test-tube. The volume of eluate was about

of blastoderms grown *in ovo* based on incubation time and on the extent of morpho-
genesis (Table III). Slightly higher values for blastoderms grown *in vitro* are found
in batches 1–5 in relation to either time or morphogenesis; the values in batches 6
and 10 are similar to the normal values, but in batches 8 and 9 they differ from
the normal.

It has thus been shown that the chick blastoderm can synthesise its own nucleic
acids to enable embryogenesis to proceed *in vitro*. Under normal conditions, in the
egg, the blastoderm probably ingests precursors of nucleic acids from the yolk, as
has been shown by the incorporation of radioactive formate[14] and thymidine[15] by
chick embryos during later stages of development. It has been suggested[2,3] that the
nucleic acids present in the yolk form a storage reserve of these precursors, which
are kept in the form of nucleic acids until degraded by enzymes in the yolk.

TABLE III

RNA/DNA RATIOS OF BLASTODERMS GROWN *in vitro*

| | | *RNA/DNA* | |
| | | *in ovo* | |
Batch No.	*in vitro*	based on incubation time	based on "corrected incubation time"
1	11.0	5 8	8.3
2	8 7	5.8	7 2
3	8 7	5.8	6 9
4	7 8	5 8	6 8
5	8 4	6 9	6.5
6	5 4	5.4	6.5
8	7 0	5.8	5.7
9	2.5	5.8	5 7
10	4 5	5.1	5 6

ACKNOWLEDGEMENTS

SUMMARY

The synthesis of RNA and DNA by explanted chick blastoderms grown on a synthetic medium
containing glucose as the sole carbon source has been demonstrated.

References p. 73.

a restricted light path on the Unicam S.P 500. Blank chromatograms were run in the same tank as the unknown; four rectangles of paper of similar size to that containing the pyrimidine were each eluted in the same manner with o 1 N HCl and then pooled to give the blank solution. The variation in absorption of the paper blanks was calculated from the absorption values at 250, 260, 280 and 300 mμ using o 1 N HCl as a blank After subtraction of the absorption at 300 mμ the absorption values for the other wavelengths were plotted against the absorption at 300 mμ (the absorption at 300 mμ increased as the weight of paper eluted) The root mean square value for deviation of optical density from a sight-fitted curve of 36 observations for each wavelength (250, 260 and 280 mμ) was equivalent to ± o o6 μg pyrimidine The purity of the pyrimidines

280/260 (urac

from ε max.

Aliquots
grease pencil

at least 10,000 counts were registered by a thin end-window counter for each sample (usually about 200 times the background). Counting errors which were experimentally determined and included plating variations, instrumental fluctuations, etc., were about six times the calculated standard deviation.

To calculate the pyrimidine content of a sample the initial specific activity (counts in min/μg) of the pyrimidine was compared with the final specific activity in the following fo m

$$Sa = S' (a + b)$$

$$b = \frac{Sa}{S'} - a$$

where $b =$ μg thymine (or uracil) in extract, $a =$ μg thymine-2-^{14}C added, $S =$ spec ... of the added thymine-2-^{14}C and $S' =$ specific activity of the final diluted th.n .. . total error (ultraviolet absorption and counting) for S' was the root mean square value two errors, and the error on b was the root mean square value of the two errors of ... uracil or thymine content of rat liver RNA and DNA was then used to relate the ... content of extracts to nucleic acid content

Colorimetric methods

Aliquots of perchloric acid extracts of RNA were neutralised and interfering hexose cor g removed by hydrolysis in 0 1 M NaOH at 100° for 15 min[21]. RNA was then determined b... th orcinol method[22] using reduced amounts of reagent so that the final volume of the isoam... al extract was only 1 ml. Values for DNA content of blastoderms and embryos determined b a modified indole method[23] based on frog testicle DNA have previously been reported[24] When RNA was present in extracts of DNA a correction was applied to deoxyribose determinations (10 μg RNA = 1 μg DNA). For comparison with the thymine isotope dilution method these ... ... with nucleic

sma albumin
dard protein.

RESULTS

Nucleic acids in the unincubated hen's egg

The "acid-soluble" fraction of homogenates of egg white and yolk contained only small amounts of free thymine or thymine nucleotide (Table I). Although there is little uracil nucleotide in the yolk, there are comparatively large amounts of free uracil or uracil nucleotides in egg white.

The nucleic acid content of egg white and yolk is given in Table II. The RNA content of the white is considerably greater than that of the yolk and there is more RNA than DNA in both white and yolk. The phenol partition method appears to give a better e ... ... m.thod and this is more pronounced in ... ... amounts of DNA appear to be extracted by perchloric acid treatment, ... s effect is

References p. 591.

REFERENCES

[1] E. HOFF-JORGENSEN, *Recent Develop Cell Physiol*, *Proc 7th Symposium Colston Resea*>⸌⸜ ⸝
Univ. Bristol, 1954, p. 79.
[2] ⸜ ⸝ ᴅ Ꮪ⸗⸗⸗⸗⸗ ᴰ⸗⸗⸗⸗⸗ ᴰ⸗⸗⸗⸗⸗ ⸗⸗⸗⸗ ⸗⸜ ⸍⸗⸗⸜⸝ 584

[6] N. T. SPRATT, *ibid.*, 110 (1949) 273
[7] V. HAMBURGER AND H. L. HAMILTON, *J Morphol*, 88 (1951) 49
[8] J. B. SOLOMON, *Biochim. Biophys Acta*, 23 (1957) 24
[9] G. CERIOTTI, *J. Biol. Chem*, 214 (1955) 59
[10] D. RAPPORT, A. CANZANELLI AND R GUILD, *Am J Physiol*, 162 (1950) 421
[11] G. CERIOTTI, *J. Biol. Chem.*, 198 (1952) 297
[12] E. W. SUTHERLAND, C. F CORI, R. HAYNES AND N S OLSEN, *ibid*, 180 (1949) ⁹.5
[13] C. H. WADDINGTON, *Nature*, 125 (1930) 924
[14] D. H. MARRIAN, A. F. W. HUGHES AND S M. WERBA, *Biochim. Biophys Acta* ᵼ⸗ ⸌
[15] M. FRIEDKIN, D. TILSON AND D. ROBERTS, *J Biol Chem*, 220 (1956) 62⁻

TABLE I

"ACID-SOLUBLE" URACIL AND THYMINE DERIVATIVES IN EGG WHITE AND YOLK

	Uracil (μg per white or yolk)	Thymine (μg per white or yolk)
White	460 ± 55	19 ± 4
Yolk	13 ± 1	66 ± 0 3

TABLE II

NUCLEIC ACIDS IN EGG WHITE AND YOLK

	RNA (μg per white or yolk)	DNA (μg per white or yolk)
Yolk		
Perchloric acid extraction	68 ± 14	52 ± 6
		57 ± 7
Phenol partition	114 ± 14	39 ± 2
White		
Perchloric acid extraction	520 ± 33	308 ± 16
Phenol partition	2260 ± 100	137 ± 30
	1595 ± 50	142 ± 5

more marked in the case of egg white than that of yolk. One explanation of the lower amounts of DNA obtained by the phenol method may be that more washing of the phenol layer with water is necessary to obtain similar extraction efficiency to that of perchloric acid. Alternatively, not all the DNA may be released from the egg proteins by treatment with p-aminosalicylate in the phenol method.

The ratio of amounts of nucleic acid in egg white and yolk (obtained by the phenol partition method) to amounts of the respective pyrimidine in the "acid-soluble" fraction is approximately constant (6.5).

Nucleic acids in the developing blastoderm and early chick embryo

The RNA content of blastoderms and developing embryos determined by the orcinol and uracil isotope dilution method during the first three days of incubation are shown in Fig. 1. It can be seen that both methods give reasonable agreement, and the increase in RNA conforms to a logarithmic growth curve. There is a more rapid synthesis of RNA in the embryo than in the developing blastoderm (embryo + vascular area).

Results obtained by colorimetric estimation of DNA[24] have been related to rat liver DNA (there was little apparent difference between analytical data on DNA obtained from either frog testicles or rat liver) and the results obtained by the deoxyribose and thymine methods are compared in Table III. The results show reasonable agreement allowing for the error on each method; the thymine results are usually slightly higher than those obtained by the deoxyribose method. A previously reported value for the amount of DNA per nucleus in early chick embryos[24] was $1.1 \cdot 10^{-6}$ μg, standard deviation ± $0 5 \cdot 10^{-6}$ μg; the average values for DNA per nucleus based on the thymine measurements are higher. In embryos, the amounts of DNA per nucleus are within the range 1.0–2.1 and in

References p. 591.

Which method is then adopted—bilateral adrenalectomy and oöphorectomy or hypophysectomy—depends largely upon the facilities at hand. There are not a great many cranial surgeons and if every case of metastasizing carcinoma of the breast was referred to them, they would find it impossible to manage them all, however hard and however fast they operated and to the exclusion of all other work. Bilateral oöphorectomy and adrenalectomy, however, can be done by competent general surgeons who can soon learn the precise technique which will yield results very nearly as good, and possibly as good, as those obtained by hypophysectomy. Sudden urgency of operation sometimes develops, especially with cranial, pulmonary and hepatic metastases. Delay from reference to a cranial surgeon's unit might be fatal, let alone the danger from time lost if the cranial surgeon considered the patient unsuitable for hypophysectomy, as well he might if she were in coma.

Essential indications for hypophysectomy are failed adrenalectomy or even the return of symptoms after relief by adrenalectomy. In these circumstances it is the only operation giving any prospect of improvement at all. Similarly, it is the operation which must be done when the tumour has progressed so far superficially or internally that exposure and removal of the adrenals or ovaries is impossible.

Lastly, the purpose of cancer surgery is to cure the early case. If a woman is admitted for radical mastectomy and finds herself in the company of garrulous and embittered hypophysectomized and adrenalectomized females, she may well, and with some justification, walk out and refuse all treatment. The organization of the ward must bear this aspect of the problem in mind and not allow the faith and hope of the early cases to be jeopardized by the example of bad results in the late.

References

Atkins, H. J. B., et al. (1952): Lancet, i, 8-

C   71.

C   7.

Cramer, W., and Horning, E. S. (1939): Lancet, i, 192.

Dao, T. L.-Y., and Huggins, C. (1955): Arch. Surg., 71, 645.

Forrest, A. P. M., and Peebles Brown, D. A. (1955): Lancet, ii,

F: . .(.   . :.   .   . .   . 11: . . .

G . .) . . (. . :   . :   (. . . .4

G . . . . ' . :. .   .   . , . . .

Hadfield, G. (1957): Lancet, i, 1058.

Huggins, C., and Bergenstal, D. M. (1952): Cancer Res., 12, 134.

I   . .   P. .   . . Ol .   . . .

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A AND B ANTIGENS OF NORMAL AND
MALIGNANT CELLS

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RECENT work has suggested that loss of antigens occurs in some forms of neoplasia (Weiler, 1956) and, according to one theory (Green, 1954), may even be the essential mechanism by which malignant transformation is brought about.

It seems pertinent, therefore, to outline as far as possible the frequency and extent of this change. For this purpose the A and B " blood group " antigens as possessed by the human urinary tract epithelium form a useful system.

The cells, both normal and neoplastic, can be easily grouped by the mixed erythrocyte epithelial cell agglutination test—hereafter referred to as the MCA test—of Coombs, Bedford and Rouillard (1956) without interference from mucus, keratin or stroma , and there occurs in the bladder a series of tumours ranging from the benign to the highly malignant.

Twenty-five tumours in patients of groups A, B and AB have been studied by the MCA test reinforced in five cases by a simple antibody inhibition technique.

METHODS

Epithelial cells unmixed with other tissues can be obtained from the bladder, ureter or renal pelvis by the simple expedient of scraping. The cells so removed are mixed in Tyrode's solution with the aid of a syringe and wide bore needle until all the clumps are broken up   In the case of tumours it may be possible to scrape enough cells off the surface . or the most peripheral parts of the tumour, which contain negligible amounts of stroma, may be snipped off with scissors. Tumours with much necrosis, infection or round-cell infiltration were excluded from the series, although the presence of a few non-tumour cells in the suspension (up to 10 per cent) was allowed.

For both agglutination and antibody-inhibition tests a cell concentration of 10,000/c. mm. is aimed at. The cells are counted in a Fuchs-Rosenthal chamber, preferably under phase-contrast, and the necessary adjustments are made. From normal tissues a high degree of accuracy can be obtained, but with tumour cells a tendency to disintegration of the cells reduces the precision.

The MCA test technique is that described by Coombs, Bedford and Rouillard (1956), the only difference being the use of human AB serum in place of their inactivated rabbit serum. Routine grouping sera were used undiluted in the ratio of one drop of cell suspension to three of serum to ensure excess of the latter.

For inhibition tests comparison with normal cells is desirable. Controls must also include the use of heterologous serum, e.g β against A cells, to determine the degree of non-specific adsorption   The results are only valid when the sus-

GALTON, D. A. G. Androgen therapy in 70 cases of advanced mammary carcinoma. *Brit. J. Cancer*, 4, 20–58 (1950). In 7, (36).

GALTON, D. A. G. Chemotherapy in the lymphomas and leukaemias. *British Practice in Radiotherapy*. Ed. by Carling, Windeyer and Smithers. 456–463 (London, Butterworth, 1955). In 10, (17).

GALTON, D. A. G. Chronic myeloid leukaemia. 1. Some observations on chronic myeloid leukaemia *Brit. J. Radiol.*, N.S., 26, 285–290 (1953). In 8, (39).

GALTON, D. A. G. Myleran in chronic myeloid leukaemia. results of treatment *Lancet*, 264, 208–213 (1953). In 8, (34).

GALTON, D. A. G. The use of myleran and similar agents in chronic leukaemias *Adv. Cancer Res* 4, 73–112 (1956). In 12, (26).

GALTON, D. A. G. The use of 6-mercaptopurine in the treatment of leukaemia *Ciba Foundation Symposium on The Chemistry and Biology of Purines*. 299–305 (London, Churchill, 1957) In 12, (66).

GALTON, D. A. G., ISRAELS, L. G., NABARRO, J. D. N., and TILL, M. Clinical trials of p. (di-2-chloroethylamino)-phenylbutyric acid (CB 1348) in malignant lymphoma *Br med J.* ii, 1172–1176 (1955). In 10, (31).

GALTON, D. A. G., and TILL, M. Myleran in chronic myeloid leukaemia. *Lancet*, 268, 42 (1955). In 10, (25).

GILBERT, I. G. F., and TAYLOR, D. M. The behaviour of zinc and radio-zinc in the rat. *Biochim biophys. Acta*, 21, 545–551 (1956). In 11, (85).

GLUCKSMANN, A., LAMERTON, L. F., and MAYNEORD, W. V. Carcinogenic effects of radiation *Cancer*, Ed. Raven. 1, 497–539 (London, Butterworth, 1957). In 12, (203).

GOLDACRE, R. J. The action of general anaesthetics on amoebae and the mechanism of the response to touch. *Symp. Soc. exp. Biol.*, No.6, 128–144 (1952). In 8, (532).

GOLDACRE, R J. Crystalline bacterial arrays and specific long-range forces. *Nature, Lond.*, 174, 732–734 (1954). In 9, (689).

GOLDACRE, R. J. The folding and unfolding of protein molecules as a basis of osmotic work. *Int. Rev. Cytol.*, 1, 135–164 (1952). In 8, (554).

GOLDACRE, R. J., EASTY, D. M., and AMBROSE, E. J. A cell compressor for the measurement of mass and concentration by interference microscopy. *Nature, Lond.*, 180, 1487–1488 (1957). In 12, (440).

GOLDACRE, R. J., and LORCH, I. J. Folding and unfolding of protein molecules in relation to cytoplasmic streaming, amoeboid movement and osmotic work. *Nature, Lond.*, 166, 497–500 (1950). In 7, (547).

GREENING, W. P. Hypophysectomy for cancer. *Proc. R. Soc. Med.*, 50, 867–868 (1957). In 12, (78).

GREENING, W. P. Irradiation of pituitary for advanced mammary cancer. *Lancet*, i, 728–729 (1956). In 11, (32).

GREENWOOD, H. H. The reactivity of aromatic bonds, with reference to carcinogenic compounds of 1:2-benzanthracene. *Brit. J. Cancer*, 5, 441–457 (1951). In 7, (556).

GREENWOOD, H. H. Some aspects of the molecular orbital theory of the chemical reactivity of aromatic molecules. *Trans. Faraday Soc.*, 48, 585–591 (1952). In 8, (584).

GUIMARAES, J. P., and LAMERTON, L. F. Further experimental observations on the late effects of thorotrast administration. *Brit. J. Cancer*, 10, 527–532 (1956). In 11, (157).

GUIMARAES, J. P., LAMERTON, L. F., and CHRISTENSEN, W. R. The late effects of thorotrast admini-

GUIMA

HADDOW, A. Advances in the study of chemical carcinogenesis. *Proc. R. Soc. Med.*, 44, 263–266 (1951). In 7, (573).

HADDOW, A. Biochemistry of cancer. *Annu. Rev. Biochem*, 24, 689–742 (1955). In 10, (689).

HADDOW, A. The chemical and genetic mechanisms of carcinogenesis. 1. Nature and mode of action. 2. Biologic alkylating agents. *The Physiopathology of Cancer*. Ed. Homburger & Fishman. 441–474, 475–551. (New York, Hoeber, 1953). In 8, (591).

HADDOW, A. Comparative studies of the biological effects of ionizing radiation and of radio-mimetic chemical agents. *Proceedings of the International Conference on the Peaceful Uses of Atomic Energy, Geneva*, 1955. (New York, United Nations, 1956). 11, 213–218. In 10, (748).

HADDOW, A. Experimental and clinical aspects of the action of various carboxylic acid derivatives in the aromatic nitrogen mustard series. *Ciba Foundation Symposium on Leukaemia Research*. 196–204. (London, Churchill, 1954). In 9, (692).

ALEXANDER, P., and STACEY, K. A. The light-scattering and non-Newtonian viscosity of high molecular weight polymethacrylic acid. *Trans. Faraday Soc.*, 51, 299–308 (1955). In 10, (336).

ALEXANDER, P., and STACEY, K. A. Production of "hidden" breaks in DNA by the direct action of ionizing radiation. *Progress in Radiobiology: proceedings of the 4th international conference on radiobiology, Cambridge,* 1955. 105–109 (Edinburgh, Oliver & Boyd, 1956). In 10, (332).

ALEXANDER, P., and TOMS, D. The effect of oxygen on the changes produced by ionizing radiations in polymers. *J. Polym. Sci.*, 22, 343–348 (1956). In 11, (255).

ALEXANDER, P., and others. Mode of action of some substances which protect against the lethal effects of x-rays. *Radiat. Res.*, 2, 392–415 (1955). In 10, (300).

ALLEN, M. J., BOYLAND, E., DUKES, C. E., HORNING, E. S., and WATSON, J. G. Cancer of the urinary bladder induced in mice with metabolites of aromatic amines and tryptophan. *Brit. J. Cancer*, 11, 212–228 (1957). In 12, (643).

AMBROSE, E. J. Fibre formation by cellular systems. *Proc. roy. Soc. B*, 148, 57–67 (1957). In 12, (398).

AMBROSE, E. J. A physical approach to the problem of cancer. *Brit. J. Cancer*, 8, 259–273 (1954). In 9, (297).

AMBROSE, E. J. The structure of chromosomes. *Progr. Biophys.*, 6, 26–55 (1956). In 11, (261).

AMBROSE, E. J. A surface contact microscope for the study of cell movements. *Nature, Lond.*, 178, 1194. In 11, (294).

AMBROSE, E. J., and BUTLER, J. A. V. Swelling and orientation phenomena with nucleoprotein films. *Disc. Faraday Soc., No.13,* 261–271 (1953). In 8, (308).

AMBROSE, E. J., and EASTY, G. C. Detection of antigen-antibody interactions by an interferometric method. *Nature, Lond.*, 172, 811 (1953). In 8, (319).

AMBROSE, E. J., and GOPAL-AYENGAR, A. R. Molecular orientation and chromosome breakage. *Heredity* 6, *suppl.*, 277–292 (1953). In 8, (320).

AMBROSE, E. J., JAMES, A. M., and LOWICK, J. H. B. Differences between the electrical charge carried by normal and homologous tumour cells. *Nature, Lond.*, 177, 576–577 (1956). In 11, (305).

ANDERSON, W., and BELCHER, E. H. A standard light source of very low intensity based on the Čerenkov effect. *Brit. J. appl. Phys.*, 5, 53–57 (1954). In 9, (121).

ANDERSON, W., MAYNEORD, W. V., and TURNER, R. C. The radon content of the atmosphere. *Nature, Lond.*, 174, 424–426 (1954). In 9, (112).

ANDERSON, W., and TURNER, R. C. Radon content of the atmosphere. *Nature, Lond.*, 178, 203–204 (1956). In 11, (45).

ASPLIN, F. D., BOYLAND, E., SARGENT, S., and WOLF, G. Hypertrophy of the testis induced by sulphadimidine and other pyrimidine derivatives. *Proc. Roy. Soc. B*, 139, 358–372 (1952). In 8, (338).

AVIS, P. G., BERGEL, F., and BRAY, R. C. Cellular constituents. The chemistry of xanthine oxidase. 1. The preparation of a crystalline xanthine oxidase from cow's milk. *J. chem. Soc.*, 1100–1105 (1955). In 10, (396).

AVIS, P. G., BERGEL, F., and BRAY, R. C. Cellular constituents. The chemistry of xanthine oxidase. 3. Estimations of the co-factors and the catalytic activities of enzyme fractions from cow's milk. *J. chem. Soc.*, 1219–1226 (1956). In 11, (346).

AVIS, P. G., BERGEL, F., BRAY, R. C., JAMES, D. W. F., and SHOOTER, K. V. Cellular constituents. The chemistry of xanthine oxidase. 2. The homogeneity of crystalline metalloflavoprotein fractions. *J. chem. Soc.*, 1212–1219. In 11, (339).

AVIS, P. G., BERGEL, F., BRAY, R. C., and SHOOTER, K. V. A crystalline material with xanthine oxidase activity. *Nature, Lond.*, 173, 1230–1231 (1954). In 9, (333).

AVIS, P. G., BERGEL, F., BRAY, R. C., and SHOOTER, K. V. Properties of purified xanthine oxidase. *Symposium on Inorganic Nitrogen Metabolism.* 552–564 (Baltimore, Johns Hopkins Pr., 1956). In 11, (326).

BARNES, J. M., et al. The non-toxicity of maleic hydrazide for mammalian tissues. *Nature, Lond.*, 180, 62–64 (1957). In 12, (929).

BARNICOT, N. A., BIRBECK, M. S. C., and CUCKOW, F. W. The electron microscopy of human hair pigments. *Ann. hum. Genet.*, 19, 231–249 (1955). In 10, (354).

BATEMAN, A. J., and SINCLAIR, W. K. Mutations induced in *Drosophila* by ingested phosphorus-32. *Nature, Lond.*, 165, 117–118 (1950). In 7, (216).

BAXTER, C. F., BELCHER, E. H., HARRISS, E. B., and LAMERTON, L. F. Anaemia and erythropoiesis in the irradiated rat: an experimental study with particular reference to techniques involving radioactive iron. *Brit. J. Haemat.*, 1, 86–103 (1955). In 10, (203).

HADDOW, A. Neoplastic diseases (cancer). *Annu. Rev. Med.*, **6**, 153–186 (1955). In 10, (655).

HADDOW, A. New facts and concepts: a general survey. *Proceedings of the 2nd Canadian Cancer Research Conference, Honey Harbour*, 1956. 361–374 (New York, Academic Pr., 1957). In 11, (764).

HADDOW, A. Our present understanding of the cancer cell. *Practitioner*, **174**, 249–258 (1955). In 10, (645).

HADDOW, A. Recent advances in the study of carcinogenesis. *S. Afr. med. J.*, **29**, 1185–1189 (1955). In 10, (743).

HADDOW, A. Recent progress in the study of chemical carcinogenesis. *Proceedings of the 2nd National Cancer Conference, Cincinnatti*, 1952. 79–82. (New York, American Cancer Society, 1954). In 9, (702).

HADDOW, A., and HORNING, E. S. Ganglioneuroma of the adrenal medulla in albino rats. *J. R. micr Soc* **70**, 181–185 (1950). In 7, (577).

HADDOW, A., HORNING, E. S., and CARLTON SMITH, N. Changes in the male accessory sex organs, and in the testes, adrenals and pituitary, associated with the growth of animal tumours. *Schweiz. med. Woch.*, **87**, *suppl. to No.14*, 1957, 396–403. In 12, (906).

HADDOW, A., and ROSS, W. C. J. Tumour growth-inhibitory alkyl sulphonates. *Nature, Lond.*, **177**, 995–996 (1956). In 11, (778).

HADDOW, A., and TIMMIS, G. M. Bifunctional sulphonic acid esters with radiomimetic activity. *Acta Un. int. Cancr.*, **7**, 469–471 (1951). In 7, (588).

HADDOW, A., and TIMMIS, G. M. Myleran in chronic myeloid leukaemia: chemical constitution and biological action. *Lancet*, **264**, 207–208 (1953). In 8, (702).

HADDOW, A., TIMMIS, G. M., and HORNING, E. S. Rhabdomyosarcomas in the albino rat. *J. R. micr. Soc.*, **74**, 59–63 (1954). In 9, (708).

HANCOCK, P. E. Thompson. The relief of pain in terminal cancer. *Practitioner*, **179**, 326–328 (1957). In 12, (82).

HARMAN, J. B., HART, P. L. de V., and LEDLIE, E. M. Treatment of polycythaemia with radio-active phosphorus. *Brit. med. J.*, **i**, 930–934 (1954). In 10, (37).

HARRIS, R. J. C. Acquired tolerance of turkeys to Rous sarcoma agent. *Proc. roy. Soc. B*, **146**, 59–66 (1956). In 11, (779).

HARRIS, R. J. C. The association of acid and alkaline phosphomonoesterase with cytoplasmic particles from chick embyro and from fowl sarcomas. *Brit. J. Cancer*, **8**, 714–721 (1954). In 9, (726).

HARRIS, R. J. C. Multiplication of Rous No.1 sarcoma agent in the chorioallantoic membrane of the embryonated egg. *Brit. J. Cancer*, **8**, 731–736 (1954). In 9, (743).

HARRIS, R. J C The phosphomonoesterase activity associated with preparations of Rous sarcoma No.1 agent. *Brit. J. Cancer*, **8**, 722–730 (1954). In 9, (734).

HARRIS, R. J. C., MALMGREN, H., and SYLVÉN, B. The polysaccharides of Rous sarcoma No.1. *Brit J. Cancer*, **8**, 141–146 (1954). In 9, (720).

HARRIS, R. J. C., ORR, S. F. D., ROE, E. M. F., and THOMAS, J. F. Investigation of polymorphism and isomerism in cytidine phosphates. *J. chem. Soc.*, 489–493 (1953). In 8, (703).

HIEGER, I. Cholesterol as a carcinogen. *Proc. roy. Soc. B.*, **147**, 84–89 (1957). In 12, (932).

HIEGER, I, and ORR, S F. D. On the carcinogenic activity of purified cholesterol. *Brit. J. Cancer*, **8**, 451–457 (1954). In 9, (749).

HODT, H. J., SINCLAIR, W. K., and SMITHERS, D. W. A gun for interstitial implantation of radio-active gold grains. *Brit. J. Radiol.*, *N.S.*, **25**, 419–421 (1952). In 8, (169).

HORNING, E. S. Endocrine factors involved in the induction, prevention and transplantation of kidney tumours in the male golden hamster. *Z. Krebsforsch.*, **61**, 1–21 (1956). In 11, (787).

HORNING, E. S Histological procedures in cancer research. *Endeavour* **13**, 207–212 (1954). In 9, (789).

HORNING, E. S. The influence of unilateral nephrectomy on the development of stilboestrol-induced renal tumours in the male hamster. *Brit. J. Cancer*, **8**, 627–634 (1954). In 9, (779).

HORNING, E. S. The local action of 20-methylcholanthrene and sex hormones on prostatic grafts. *Brit J. Cancer*, **6**, 80–88 (1952). In 8, (708).

HORNING, E. S. Observations on hormone-dependent renal tumours in the golden hamster. *Brit. J. Cancer*, **10**, 678–687 (1956). In 11, (808).

HORNING, E. S Some anomalies in endocrine carcinogenesis. *Lect. scient. Basis Med.*, **5**, 421–438 (1957). In 12 (939).

HORNING, E. S. Studies on the induction of lung cancer in mice. *Brit. J. Cancer*, **4**, 235–244 (1950). In 7, (591).

BEALE, R. N. Proximity effects in the interpretation of the basic strengths of primary aromatic amines. *J. chem. Soc.*, 4494–4499 (1954). In 9, (890).

BEALE, R. N., HARRIS, R. J. C., and ROE, E. M. F. The nucleic acids of normal and tumour tissues. The preparation and composition of a pentonucleic acid from the fowl sarcoma No R C H 15. *J. chem. Soc.*, 1397–1408 (1950). In 7, (340)

BEALE, R. N., HARRIS, R. J. C., and ROE, E. M. F. The nucleic acids of normal and tumour tissues. 2. The preparation and composition of pentonucleic acids from the fowl tumours Rous No.1, and Duran-Reynals "D". *J. chem. Soc.*, 1034–1038 (1952). In 8, (341)

BEALE, R. N., and ROE, E. M. F. The spectrophotometric determination of steric hindrance in some stilbene derivatives. *J. Amer. chem. Soc*, 74, 2302–2307 (1952). In 8, (342)

BEALE, R. N., and ROE, E. M. F. The spectrophotometric determination of steric hindrance in stilbene derivatives. *Photoelect. Spectr. Gr. Bull*, No.7, 155–159 (1954). In 9, (343)

BEALE, R. N., and ROE, E. M. F. Ultra-violet absorption spectra of some metabolites of anthracene and phenanthrene. *J. chem. Soc.*, 2884–2888 (1951). In 7, (344)

BEALE, R. N., and ROE, E. M. F. Ultra-violet absorption spectra of *trans*-stilbenes and their derivatives. Part I. *trans*- and *cis*-stilbenes. *J. chem. Soc*, 2755–2763 (1953). In 8, (345)

BELCHER, E. H. Abnormal efficiencies in the scintillation-counting of gamma rays. *Nucleonics*, 166, 742. (1950). In 7, (220).

BELCHER, E. H. The density, granularity and silver content of photographic film exposed to radiations. *J. photogr. Sci.*, 1, 86–89 (1953). In 8, (91).

BELCHER, E. H. The luminescence of irradiated transparent media and the Čerenkov effect. 1. The luminescence of aqueous solutions of radioactive isotopes. *Proc. roy. Soc. A*, 216, 90–102 (1953). In 8, (64)

BELCHER, E. H. Measurement of gamma-ray energies with the scintillation counter. *Nature, Lond.*, 166, 826 (1950). In 7, (223).

BELCHER, E. H. Radiation dosimetry with scintillation detectors. *Brit. J. Radiol., N.S*, 26, 455–464 (1953). In 8, (81).

BELCHER, E. H. Scintillation counters using liquid luminescent media for absolute standardization and radioactive assay. *J. scient. Instrum*, 30, 286–289 (1953). In 8, (77).

BELCHER, E. H. Scintillations produced in liquids by high-energy radiation *aa* dinaphthyl as a scintillating medium. *Nature, Lond.*, 167, 314 (1951). In 7, (225).

BELCHER, E. H., and EVANS, H. D. A directional scintillation counter for clinical measurements. *J. scient. Instrum.*, 28, 71–75 (1951). In 7, (227).

BELCHER, E. H., and EVANS, H. D. The distribution of cerebral tumours with radioactive derivatives of fluorescein. physical limit 272–279 (1951). In 7, (232).

BELCHER, E. H., and GEILINGER, J. radiation dosimetry. *Brit. J. Radiol., N.S.*, 30, 103–105 (1957) In 12, (200).

BELCHER, E. H., GILBERT, I. G. F., and LAMERTON, L. F Experimental studies with radioactive iron. *Brit. J. Radiol., N.S.*, 27, 387–392 (1954) In 9, (115).

BELCHER, E. H., and HARRISS, E. B Radioactive tracer investigations of red cell survival in the rat. *2nd Gastemer internationalen Symposion, Badgastein*, 1956. (München, Urban & Schwarzenberg, 1956) 5–11. In 11, (46)

BELCHER, E. H., and HARRISS, E. B. Studies of plasma volume, red cell volume and total blood volume in young growing rats. *J. Physiol.*, 139, 64–78 (1957). In 12, (246).

BELCHER, E. H., HARRISS, E. B, and LAMERTON, L. F. Effects of radiation on erythropoiesis in the rat: turnover studies using radioactive iron. *Progress in Radiobiology: proceedings of the 4th international conference on radiobiology, Cambridge*, 1955. (Edinburgh, Oliver & Boyd, 1956) 303–307. In 10, (221).

BELCHER Radioactive isotopes in medical diagnosis. *Progr. nucl.* In 11, (53).

BELCHER density, granularity and silver content of photographic *J. photogr. Sci.*, 1, 86–89 (1953). In 8, (91).

BERGEL, F. Attempts at chemotherapy of malignant diseases. *J. Pharm., Lond.*, 7, 297–313 (1955). In 10, (379).

BERGEL, F. Chemistry in cancer research. *Med. Pr.*, 232, 214–219 (1954). In 9, (318).

BERGEL, F. Some chemical aspects of abnormal growth. *Nature, Lond.*, 174, 156–159 (1954). In 9, (315).

BERGEL, F. Some chemical aspects of abnormal growth. *Lect. scient. Basis Med.*, 4, 54–73 (1956). In 11, (306).

BERGEL, F. Some chemical aspects of normal growth. *Nature, Lond.*, 171, 905–908 (1953). In 8, (374).

HORNING, E. S., and WHITTICK, J. W. The histogenesis of stilboestrol-induced renal tumours in the male golden hamster. *Brit. J. Cancer*, **8**, 451–457 (1954). In 9, (767).

HUNT, A. H. Carcinoma of the maxilla. *British Surgical Progress, 1956*. 33–46. (London, Butterworth, 1956). In 11, (7).

HUNT, A. H. Discussion on the treatment of advanced cancer of the rectum. *Proc. R. Soc. Med.*, **43**, 1086–1087 (1950). In 7, (76).

HUNT, A. H. Hypophysectomy and adrenalectomy in the treatment of cancer of the breast. *Practitioner*, **179**, 256–262 (1957). In 12, (85).

HUNT, A. H. Portal vein thrombosis. *Med. ill*, **10**, 89–99 (1956). In 11, (21)

HUNT, A. H. Terebrant rodent ulcer with widespread blood-borne metastases. *Br. J. Surg.*, 151–153 (1952). In 8, (45).

HUNTER, G. D., and BUTLER, J. A. V. Stimulation by ribonucleic acid of induced *beta-galactosidase* formation in *Bacillus megaterium*. *Biochim. biophys. Acta*, **20**, 405–406 (1956). In 11, (...).

HUNTER, G. D., CRATHORN, A. R., and BUTLER, J. A. V. Sites of the incorporation of *amino* acid into proteins of *Bacillus megaterium*. *Nature, Lond.*, **180**, 383–384 (1957). In (...).

IRELAND, H. J. D. Radiography with radioactive isotopes. *Practitioner*, **178**, 447. In 12, (261).

KAY, H. E. M. A and B antigens of normal and malignant cells *Brit. J. Cancer*, **11**, 401–411 (1957). In 12, (92).

KEANE, B. E., and SPIEGLER, G. Stray radiation from diagnostic x-ray beams. *Brit. J. Radiol.* *N.S.*, **24**, 198–203 (1954). In 7, (240).

KING, R. J. A phase-contrast searcher for the Burch reflecting microscope. *Nature, Lond.*, **173**, 220–221 (1954). In 9, (879).

KING, R. J., and ROE, E. M. F. Optical conditions for quantitative ultraviolet microspectroscopy. *J. R. micr. Soc.*, **73**, 82–93 (1953). In 8, (722).

KING, R. J., and ROE, E. M. F. Photomicrography with the Burch reflecting microscope. *J. R. micr. Soc.*, **74**, 64–68 (1954). In 9, (878).

KING, R. J., and ROE, E. M. F. Ultraviolet irradiation of leucocytes. *Proceedings of the 1st International Photobiological Congress, Amsterdam, 1954*. 149–151 (Wageningen, Veenman & Zonen, 1956). In 9, (874).

KING, R. J., and ROE, E. M. F. Ultra-violet micrography of the tumour and virus of Rous fowl sarcoma. *Photogr. J.*, **90B**, 94–97 (1950). In 7, (604)

KIRBY, K. S. A new method for the isolation of ribonucleic acids from mammalian tissues. *Biochem. J.*, **64**, 405–408 (1956). In 11, (573).

KIRBY, K. S. A new method for the isolation of deoxyribonucleic acids; evidence on the nature of bonds between deoxyribonucleic acid and protein. *Biochem. J.*, **66**, 495–504 (1957). In 12, (750).

KOLLER, P. C. Chromosome breakage. *Progr. Biophys.*, **4**, 195–243 (1954). In 9, (795).

KOLLER, P. C. The cytological effects of irradiation at low intensities. *Heredity* **6**, *suppl.*, 5–22 (1953). In 8, (736).

KOLLER, P. C. Cytological variability in human carcinomatosis. *Ann. N. Y. Acad. Sci.*, **63**, 793–816 (1956). In 11, (829).

KOLLER, P. C. Dicentric chromosomes in a rat tumour induced by an aromatic nitrogen mustard. *Heredity* **6**, *suppl.*, 181–196 (1953). In 8, (754).

KOLLER, P. C. Mitotic poisoning by 4-aminostilbenes. *J. nat. Cancer Inst.*, **15**, 1237–1264 (1955). In 10, (754).

KOLLER, P. C. The genetic component of cancer. *Cancer*. Ed. Raven. 1, 335–403 (London, Butterworth, 1957). In 12, (957).

KOLLER, P. C. The role and importance of mutation, variation and adaptation in malignant growth. *Acta genet.*, **6**, 283–290 (1956). In 11, (821)

KOLLER, P. C., and VERONESI, U. The effects of a nitrogen mustard derivative of phenylalanine on experimental tumours. *Brit. J. Cancer*, **10**, 703–714 (1956). In 11, (853).

KON, G. A. R., and ROBERTS, J. J. Some compounds related to the aromatic "nitrogen mustards." *J. chem. Soc.*, 978–981 (1950). In 7, (608).

KRAMER, S., BURTON, L. K., and TROTT, N. G. Radioactive isotopes in the localization of brain tumours. *Acta radiol., Stockh.*, **46**, 415–424 (1956). In 11, (92).

KRAMER, S., CONCANNON, J. P., EVANS, H. D., and CLARK, G. M. Thyroid carcinoma a report on the diagnostic and therapeutic use of radioiodine. *Brit. J. Radiol., N.S.*, **28**, 307–313 (1955). In 10, (100).

LAMERTON, L. F. Some aspects of the problem of dosage estimation in x-ray treatment. *Brit. J. Radiol., N.S.*, **24**, 385–388 (1951). In 7, (246).

BERGEL, F. The study of biologically active agents as a vocation. *Chem. & Ind. (Rev.).* 740–750 (1957). In 12, (441).

BERGEL, F. Synthesis of 4-amino-3-methoxy-stilbene. *Chem. & Ind. (Rev.)* 904–905 (1954). In 9, (324).

BERGEL, F., and BRAY, R. C. Stabilization of xanthine oxidase activity by salicylate. *Nature, Lond.,* 178, 88–89 (1956). In 11, (353).

BERGEL, F., BRAY, R. C., HADDOW, A., and LEWIN, I. Enzymic control of purines by xanthine oxidase. *Ciba Foundation Symposium on the Chemistry and Biology of Purines.* 256–266 (London, Churchill, 1957). In 12, (534).

BERGEL, F., BRAY, R. C., and HARRAP, K. R. Die Trennung von Riboflavin-derivaten durch Gegenstromverteilung. *Liebigs Ann.,* 607, 219–224 (1957). In 12, (461).

BERGEL, F., BURNOP, V. C. E., and STOCK, J. A. Cyto-active amino-acids and peptides. 2. Resolution of *para*-substituted phenylalanines and synthesis of *p*-di-(2-chloroethyl) amino-DL-phenyl [*beta*-14C] alanine. *J. chem. Soc.,* 1223–1230 (1955). In 10, (403).

BERGEL, F., EVERETT, J. L., MARTIN, J. B., and WEBB, J. S. Cellular constituents. Major and minor metals in normal and abnormal tissues. 1. Analysis of Wistar rat livers for copper, iron, magnesium, manganese, molybdenum, and zinc. *J. Pharm., Lond.,* 9, 522–531 (1957). In 12, (515).

BERGEL, F., EVERETT, J. L., ROBERTS, J. J. and Ross, W. C. J. Aryl-2-halogenoalkylamines. 15 Some cationic and basically substituted aryl compounds. *J. chem. Soc.,* 3835–3839 (1955). In 10, (432).

BERGEL, F., and LEWIS, G. E. Cyto-active amino-acids and peptides. 3. Synthesis of *para*-substituted phenyl and alkoxymethyl ethers of tyrosine. *J. chem. Soc.,* 1816–1819 (1957). In 12, (452).

BERGEL, F., and STOCK, J. A. Cyto-active amino-acids and peptides. 4. Synthesis of α-[*p*-Di-(chloroethyl)amino-benzylidene- and -benzyl-]hippuramides and -hippuramido-acids. *J. chem. Soc.,* 4563–4567 (1957). In 12, (456).

BERGEL, F., and LEWIS, G. E. Polarimetric changes of optically active amino-acid derivatives and amines in the presence of ketones. *Chem. & Ind. (Rev.).,* 774–775 (1955). In 10, (474).

BERGEL, F., and STOCK, J. A. Cyto-active amino-acid and peptide derivative. 1. Substituted phenylalanines *J. chem. Soc.,* 2409–2417 (1954). In 9, (325).

BIDSTRUP, P. L., and CASE, R. A. M. Carcinoma of the lung in workmen in the bichromates-producing industry in Great Britain. *Brit. J. industr. Med.,* 13, 260–264 (1956). In 11, (620).

BIGNALL, J. R. Bronchial carcinoma. *Lancet,* i, 768–790 (1955). In 10, (1).

BIGNALL, J. R. Bronchial carcinoma effect of radiotherapy on survival. *Lancet,* i, 876–879 (1956). In 11, (1).

BIGNALL, J. R. Natural duration of bronchial carcinoma. *Lancet,* i, 210–214 (1955). In 10, (5).

BIGNALL, J. R., and MOON, A. J. Survival after lung resection for bronchial carcinoma. *Thorax,* 10, 183–190 (1955). In 10, (9).

BIRBECK, M. S. C., and MERCER, E. H. Cell membranes and morphogenesis. *Nature, Lond.,* 178, 985–986 (1956). In 11, (409).

BIRBECK, M. S. C., and MERCER, E. H. Electron microscopic, X-ray and birefringence studies on the proteins of the hair follicle. *1st European conference on electron microscopy, Stockholm,* 1956. 158–160. (Stockholm, Almqvist & Wiksell, 1957). In 12, (602).

BIRBECK, M. S. C., and MERCER, E. H. .... the human hair follicle. 1. Introduction and the hair cortex .. .. . : . (547).

BIRBECK, M. S. C., and MERCER, E. H. . .. .le. 2. The hair cuticle. *J. biophys. biochem.* . . . .

BIRBECK, M. S. C., and MERCER, E. H. The electron microscopy of the human hair follicle. 3. The inner root sheath and trichohyaline. *J. biophys. biochem. Cytol.,* 3, 223–230 (1957) In 12, (581).

BIRBECK, M. S. C., MERCER, E. H., and BARNICOT, N. A. The structure and formation of pigment granules in human hair. *Exp. Cell Res.,* 10, 505–514 (1956). In 11, (396)

BIRBEC ....morphogenesis *1st European* ist & Wiksell,

BIRBEC . . . l preparation of cellul: .... *1st European conference on electron microscopy,* ...kholm, 1956. 125–127 .) In 12, (596)

BIRBECK, M. S. .... improved medium for the isolation of liver mitochor ....)–624 (1956). In 11, (373)

LAMERTON, L. F., and BAXTER, C. F. An experimental study of radiation-induced anaemia with reference to shielding procedure and platelet changes. *Brit. J. Radiol.*, N.S., 28, 87–94 (1955). In 10, (227).

LAMERTON, L. F., and BELCHER, E. H. The modification of the radiation response by shielding procedures. *Proceedings of the 2nd Radiobiology Symposium, Liège*, 1954. 136–140. (London, Butterworth, 1955). In 9, (139).

LAMERTON, L. F., BELCHER, E. H., and HARRISS, E. B. Experimental studies in normal and irradiated rats using radioactive iron. *Proceedings of the 2nd Radioisotopes Conference, Oxford*, 1954. 210–218 (London, Butterworth, 1954). In 9, (144).

LAMERTON, L. F., BELCHER, E. H., and HARRISS, E. B. Radioactive iron studies in normal and irradiated rats. *Proc. R. Soc. Med.*, 49, 863–868 (1956). In 11, (102).

LAMERTON, L. F., ELSON, L. A., and CHRISTENSEN, W. R. A study of the phases of radiation response in the rat. 1. The effects of uniform whole body irradiation. *Brit. J. Radiol.*, N.S., 26, 510–518 (1956). In 8, (98).

LAMERTON, L. F., ELSON, and HARRISS, E. B. A study of the phases of radiation response in the rat. 2. The effects of non-uniform irradiation. *Brit. J. Radiol.*, N.S., 26, 568–576 (1953). In 8, (107).

LAMERTON, L. F., and HARRISS, E. B. Some autoradiographic studies of non-uniform distribution of radioactive phosphorus in tissues. *Brit. med. J.*, ii, 932–935 (1951). In 7, (250).

LAMERTON, L. F., and WINSBOROUGH, M. An approach to the problem of scattering of high voltage radiation in non-homogeneous and limited media. *Brit. J. Radiol.*, N.S., 23, 236–244 (1950). In 7, (256).

LAWLEY, P. D. The hydrolysis of methylated deoxyguanylic acid at pH7 to yield 7-methylguanine. *Proc. chem. Soc.*, 290–291 (1957). In 12, (1047).

LAWLEY, P. D. Interaction studies with DNA. 1. The binding of rosaniline at low ratio of concentrations rosaniline: DNA, and competitive effect of sodium and other metal cations. *Biochim. biophys. Acta*, 19, 160–167 (1956). In 11, (867).

LAWLEY, P. D. Interaction studies with DNA. 2. The binding of "dimeric" and "polymeric" rosaniline by sodium thymonucleate and the metachromatic effect. *Biochim. biophys. Acta*, 19, 328–332 (1956). In 11, (875).

LAWLE ⠄⠄ ⠄⠄ ⠄⠄

LAWLEY, P. D. Interaction studies with DNA. 4. The binding of 5-aminoacridine studied fluorimetrically, and its comparison with the binding of rosaniline. *Biochim. biophys. Acta*, 22, 451–458 (1956). In 11, (888).

LAWLEY, P. D. The relative reactivities of deoxyribonucleotides and of the bases of DNA towards alkylating agents. *Biochim. biophys. Acta*, 26, 450–451 (1957). In 12, (1045).

LAWLEY, P. D., and WALLICK, C. A. The action of alkylating agents on deoxyribonucleic acid and guanylic acid. *Chem. & Ind.* (*Rev.*), 633 (1957). In 12, (1049).

LAYNE, D. A., LOGUE, V., MAYNEORD, W. V., McKISSOCK, W., and SMITHERS, D. W. The treatment of cerebral gliomas with 24-million-volt x rays. *Lancet*, i, 516–519 (1953). In 8, (172).

LEDERMAN, M. Bucco-pharyngeal cancer. A clinical study with special reference to "sulcus tumours" and classification. *Brit. J. Radiol.*, N.S., 29, 536–543, 605–622, 673–678, (1956). In 11, (165).

LEDERMAN, M. Cancer of the laryngopharynx. *J. Laryng.*, 68, 333–369 (1954). In 9, (13).

LEDERMAN, M. Cancer of the larynx classification. technique and results of teleradium treatment. *Rep. Inst. Laryng. Otol.*, 1, 184–187 (1951). In 7, (173).

LEDERMAN, M. Cancer of the pharyngo-laryngeal groove with special reference to sinus pyriformis. *J. Laryng.*, 67, 641–654 (1953). In 8, (186).

LEDERMAN, M. Cancers de l'hypopharynx. Classification. Resultats de la radiothérapie. *Ann. Oto-laryng.*, 72, 506–527 (1955). In 10, (107).

LEDERMAN, M. The classification and staging of cancer of the larynx. *Brit. J. Radiol.*, N.S., 25, 462–471 (1952). In 8, (176).

LEDERMAN, M. Radiothérapie des ulcères de la cornée. *J. belge. Radiol.*, 34, 450–460 (1951). In 7, (177).

LEDERMAN, M. Radiotherapy of cancer of the penis. *Brit. J. Urol.*, 25, 224–232 (1953). In 8, (200).

LEDERMAN, M. Radiotherapy of non-malignant diseases of the eye. *Brit. J. Ophthal.*, 41, 1–19 (1957). In 12, (98).

BIRD, M. J. Chemical production of mutations in *Drosophila:* comparison of techniques. *J. Genet.*, 50, 480–485 (1952). In 8, (377).

BIRD, M. J. Production of mutations in *Drosophila* using four aryl-2-halogenoalkylamines *Nature, Lond.*, 165, 491–2 (1950). In 7, (357).

BIRD, M. J., and FAHMY, O. G. Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. 1. 1;2, 3;4-diepoxybutane. *Proc. roy. Soc B*, 140, 556–578. In 8, (383).

BLAIKLEY. J. B. Pelvic exenteration. *Proc. R. Soc Med.*, 46, 753–757 (1953). In 8, (1)

BLAIKLEY, J. B., LEDERMAN, M., and LEWIS, T. L. T. Results of treatment of cancer of the cervix *Lancet*, ii, 950–1 (1952). In 8, (163)

BLAIKLEY, J. B., LEDERMAN, M., and SIMMONS, C. A. Carcinoma of the cervix at Chelsea Hospital for Women 1933–56; five-year results of treatment 1933–51. *Lancet*, ii, 1320–13 3 (1957). In 12, (21).

BLONDAL, H. A plasma I131 index for assessing thyroid activity. *Brit. J. Radiol*, N.S. 25, 193 2(2 (1952). In 8, (95).

BLOOM, H. J. G., CROCKETT, D. J., ERINS, R. P., LEDLIE, E. M., TAYLOR, D. M., and VARSON, P. S. The stability of radioactive iodinated serum albumin preparations in man *Brit. J. Radiol.* N.S., 29, 585–587 (1956). In 11, (147).

BOOTH, J., and BOYLAND, E. The biochemistry of aromatic amines. 3. Enzymic hydroxylation of rat-liver microsomes. *Biochem. J.*, 66, 73–78 (1957). In 12, (670)

BOOTH, J., and BOYLAND, E. The reaction of the carcinogenic dibenzcarbazoles and acridines with purines and nucleic acid. *Biochim. biophys. Acta*, 12, 75 (1953) In 8, (397)

BOOTH, J., BOYLAND, E., and ORR, S. F. D. A spectroscopic study of the nature of the complexes of purines with aromatic compounds. *J. chem. Soc.*, 598–603 (1954). In 9, (440)

BOOTH, J., BOYLAND, E., and MANSON, D. Metabolism of polycyclic compounds 9 Metabolism of 2-naphthylamine in rat tissue slices. *Biochem. J*, 60, 62–71 (1955). In 10, (504).

BOOTH, J., BOYLAND, E., and TURNER, E. E. The optical resolution of (±)trans-9 10-dihydroxy-9:10-dihydrophenanthrene. *J. chem Soc.*, 2808–2809 (1950). In 7, (361)

BOOTH, J., BOYLAND, E., and TURNER, E. E. The reduction of o-quinones with lithium aluminium hydride. *J. chem. Soc.*, 1188–1190 (1950). In 7, (363)

BOYLAND, E. The biochemistry of abnormalities in cell division. *Biochim. biophys. Acta*, 4, 293–300 (1950). In 7, (366).

BOYLAND, E. The biological significance of metabolism of polycyclic compounds. *Biochem Soc. Symp. No.5*, 40–54 (1950). In 7, (374)

BOYLAND, E. La carcinogénèse et le cancer chez l'homme. *Bull. Soc. Chim. biol., Paris*, 38, 827–842. In 11, (411).

BOYLAND, E. Chemotherapy of cancer. *Lect. scient. Basis Med.*, 3, 327–346 (1954). In 9, (376).

BOYLAND, E. The determination of carcinogenic activity *Acta Un. int. Cancr.*, 13, 271–278 (1957). In 12, (613).

Bo~

Bo~

BOYLAND, E. Mutagens. *Pharmacol. Rev.*, 6, 345–364 (1954). In 9, (396).

BOYLAND, E. Possibilities of the endogenous origin of carcinogens. *Zweites Freiburger Symposion uber Grundlagen und Praxis chemischer Tumorbehandlung, 1953. 63–58* (Berlin, Springer, 1953). In 9, (416).

BOYLAND, E., BUTLER, L. O., and CONWAY, B. E. The electrophoretic behaviour of normal and pathological human sera in relation to the polarographic serum test for cancer. *Brit. J. Cancer*, 5, 235–243 (1951). In 7, (400)

BOYLAND, E., GASSON, J. E., and WILLIAMS, D. C. Enzyme activity in relation to cancer. *Brit. J. Cancer*, 11, 120–129 (1957). In 12, (621).

BOYLAND, E., GASSON, J E., and WILLIAMS, D. C. 5-hydroxytryptamine excretion in patients with carcinoma of the larynx and bronchus. *Lancet* ii, 975–976 (1954). In 11, (446).

BOYLAND, E., GOSS, G. C. L., and WILLIAMS-ASHMAN, H. G. The hexokinase activity of animal tumours. *Biochem. J.*, 49, 321–325 (1951). In 7, (409).

BOYLAND, E., HARRIS, J., and HORNING, E. S. The induction of carcinoma of the bladder in rats with acetamidofluorene. *Brit. J. Cancer* 8, 647–654 (1954). In 9, (422).

BOYLAND, E., and KOLLER, P. C. Effects of urethane on mitosis in the Walker rat carcinoma. *Brit. J. Cancer*, 8, 677–684 (1954). In 9, (431).

BOYLAND, E., and MANSON, D. The biochemistry of aromatic amines. 4. O-glucosiduronic acid derivatives of 2-naphthylamine. *Biochem. J.*, 67, 275–279 (1957). In 12, (676).

LEDER? ˙˙ ˝ ˝ ˙ · ˙ ˙ic diseases of the eye. *Acta Concilian Opthhal-* In 7, (188).

LEDER? · lignant melanomata. *Trans. ophthalm Soc., L K,*

LEDERMAN, M. Radiotherapy in the treatment of orbital tumours. *Brit. J. Ophthal.* 40, 592 610 (1956). In 11, (197).

LEDERMAN, M. Some applications of radioactive isotopes in ophthalmology. *Brit J Radiol.,* N.S., 29, 1–13 (1956). In 11, (123).

LEDERMAN, M. Some clinical applications in radium therapy of a probe type direct reading dosage rate meter: a preliminary report. *Brit. J Radiol.,* N.S., 23, 430–436 (1950) ? ?, ?.

LEDERMAN, M. Technique of radiation treatment of orbital tumours. *Brit. J. Radiol* N ??? 469–476 (1957). In 12, (117).

LEDERMAN, M. Therapy in cancer of the uterine cervix. *Acta Un. int. Cancr.,* 10, ??? ?,?? In 9, (50).

LEDLIE, E. M. A comparative analysis of the incidence and of the results of treatment of ??? ?? *Brit. J. Radiol.,* N.S., 26, 290–295 (1953). In 8, (48).

LEDLIE, E. M., and BAXTER, C. F. Some clinical applications of techniques with ?, ?? 2??? of 59-Fe. *Proceedings of the 2nd Radioisotope Conference, Oxford,* 1954. ?? ??? ??????, Butterworth, 1954). In 9, (58).

LEDLIE, E. M., and HARMER, M. H. Cancer of the mouth: a report on 800 cases. *Brit. J C ???* 4, 6–19 (1950). In 7, (78).

LEDLIE, R. C. B., and SMITHERS, D. W. Carcinoma of the penis in a man circumcised in infancy. *J. Urol.,* 76, 756–757 (1956). In 11, (227).

LEDOUX, L. Action of ribonuclease on certain ascites tumours. *Nature, Lond.,* 175, 258–259 (1955). In 10, (782).

LEDOUX, L. Action of ribonuclease on two solid tumours *in vivo. Nature, Lond.,* 176, 36–37 (1955). In 10, (783).

LEDOUX, L., and REVELL, S. H. Action of ribonuclease on neoplastic growth. 1. Chemical aspects of normal tumour growth: the Landschütz ascites tumour. *Biochim. biophys. Acta,* 18, 416–426 (1955). In 10, (785).

LEWIN, I. Xanthine oxidase activity in normal and abnormal growth. *Proc. R. Soc. Med.,* 50, 563–570 (1957). In 12, (470).

LEWIN, I., LEWIN, R., and BRAY, R. C. Xanthine oxidase activity during mammary carcinogenesis in mice. *Nature, Lond.,* 180, 763–764 (1957). In 12, (478).

LOOSEMORE, T. G. E., and WHITTICK, J. W. Gross tumour embolism of the axillary artery by ˙ ˙˙ ˙ ˙ ˙ ? D??? D??? 63, 133, 138 (1951). In 7, (92).

LOVELE · ˙ · ˙˙ ˙ · n the study of chromosome breakage ˙˙ ˙˙ · ? ? ? *Heredity* 6, *suppl.,* 293–298 (1953). In 8, (774).

LOVELESS, A. Qualitative aspects of the chemistry and biology of radiomimetic (*mutagenic*) substances. *Nature, Lond.,* 167, 338–342 (1951). In 7, (612).

LUCY, J. A., and BUTLER, J. A. V. Fractionation of deoxyribonucleoprotein. *Bull. Soc. chim. Belg.,* 65, 133–139 (1956). In 11, (539).

LUCY, J. A., and BUTLER, J. A. V. Fractionation of deoxyribonucleoprotein by successive extraction with constant salt concentration. *Nature, Lond.,* 174, 32–33 (1954). In 9, (602).

MACKAY, N. R. Radioactive colloidal gold in the treatment of pleural and peritoneal effusions of malignant origin: review of 235 cases. *Lancet,* ii, 761–764 (1957). In 12, (125).

MACKAY, N. R. Radioactive isotopes in therapy. *Practitioner,* 178, 431–439 (1957). In 12, (129).

MACKAY, N. R. Tolerance of the bladder to intracavitary irradiation. *J. Urol.,* 76, 396–400 (1956). In 11, (229).

MAYNEORD, W. V. Applications of physics in the diagnosis and treatment of cancer. *Nature, Lond.,* 168, 1023–1025 (1951). In 7, (265).

MAYNEORD, W. V. Clinical application of radio-active isotopes. *Med. Pr.,* 230, 294–297 (1953). In 8, (128).

MAYNEORD, W. V. Modern radiation hazards in clinical practice. *Brit. J. Radiol.,* N.S., 24, 6–11 (1951). In 7, (274).

MAYNEORD, W. V. Radiography of the human body with radioactive isotopes. *Lancet,* i, 276–278 (1952). In 8, (116).

MAYNEORD, W. V. The radiography of the human body with radioactive isotopes. *Brit. J. Radiol.,* N.S., 25, 517–525 (1952). In 8, (119).

BOYLAND, E., and MANSON, D. The oxidation of aromatic amines. 5. Oxidation by perphosphoric acids. *J. chem. Soc.*, 4689–4694 (1957). In 12, (684).

BOYLAND, E., MANSON, D., and ORR, S. F. D. The biochemistry of the aromatic amines. 2. The conversion of arylamines into arylsulphamic acids and arylamine-*N*-glucosiduronic acids. *Biochem. J.*, 65, 417–423 (1957). In 12, (663).

BOYLAND, E., MANSON, D., and SIMS, P. The preparation of *o*-aminophenyl sulphates. *J. chem. Soc.*, 3623–3627 (1953). In 8, (425).

BOYLAND, E., MANSON, D., SIMS, P., and WILLIAMS, D. C. The biochemistry of aromatic amines. The resistance of some *o*-aminoaryl sulphates to hydrolysis by aryl sulphatases. *Biochem. J.* 62, 68–71 (1956). In 11, (427).

BOYLAND, E., and SARGENT, S. The local greying of hair in mice treated with x rays and radiomimetic drugs. *Brit. J. Cancer*, 5, 433–440 (1951). In 7, (414).

BOYLAND, E., and SIMS, P. Metabolism of polycyclic compounds. 11. The conversion of naphthalene into 2-hydroxy-1-naphthyl sulphate in the rabbit. *Biochem. J.*, 66, 38–40 (1957). In 12, (681).

BOYLAND, E., and SIMS, P. The action of some free radicals on naphthalene. *J. chem. Soc.*, 2966–2972 (1953). In 8, (430).

BOYLAND, E., and SIMS, P. The oxidation of some aromatic amines with persulphate. *J. chem. Soc.*, 980–985 (1954). In 9, (446).

BOYLAND, E., SIMS, P., and WILLIAMS, D. C. The oxidation of tryptophan and some related compounds with persulphate. *Biochem. J.*, 62, 546–550 (1956). In 11, (431).

BOYLAND, E., and SOLOMON, J. B. Metabolism of polycyclic compounds. 8. Acid-labile precursors of naphthalene produced as metabolites of naphthalene. *Biochem. J.*, 59, 518–522 (1955). In 10, (499).

BOYLAND, E., and SOLOMON, J. B. Metabolism of polycyclic compounds. 10. Estimation of metabolites of naphthalene by paper chromatography. *Biochem. J.*, 63, 679–683 (1956). In 11, (441).

BOYLAND, E., WALLACE, D. M., and WILLIAMS, D. C. The activity of the enzymes sulphatase and *beta*-glucuronidase in the urine, serum and bladder tissue. *Brit. J. Cancer*, 9, 62–79 (1955) In 10, (481).

BOYLAND, E., WALLACE, D. M., and WILLIAMS, D. C. Enzyme activity in relation to cancer: inhibition of urinary *beta*-glucuronidase of patients with cancer of the bladder by oral administration of 1 4-saccharolactone and related compounds. *Brit. J. Cancer*, 11, 578–589 (1957). In 12, (631).

BOYLAND, E., and WATSON, G. 3-hydroxyanthranilic acid, a carcinogen produced by endogenous metabolism. *Nature, Lond.*, 177, 837–838 (1956). In 11, (450).

BOYLAND, E., and WILLIAMS, D. C. The metabolism of tryptophan. 2. The metabolism of tryptophan in patients suffering from cancer of the bladder. *Biochem. J.*, 64, 578–582 (1956). In 11, (436).

BOYLAND, E., and WILLIAMS, D. C. Tryptophan metabolism in patients with carcinoid and other tumours. *Proc. R. Soc. Med*, 50, 451–452 (1957). In 12, (690).

BOYLAND, E., and WILTSHIRE, G. H. Metabolism of naphthalene by liver slices. *Biochem. J.*, 53, 424–426 (1953) In 8, (437).

BOYLAND, E., and WILTSHIRE, G. H. Metabolism of polycyclic compounds. 7. The metabolism of naphthalene, 1-naphthol and 1:2-dihydroxy-1:2-dihydronaphthalene by animals. *Biochem. J.*, 53, 636–641 (1953). In 8, (440).

BOYLAND, E., and WOLF, G. Metabolism of polycyclic compounds. 6. Conversion of phenanthrene into dihydroxydihydro phenanthrenes. *Biochem. J*, 47, 64 (1950). In 7, (424).

BROOKS, W. D., DAVIDSON, M., PRICE THOMAS, C., ROBSON, K., and SMITHERS, D. W. Carcinoma of the bronchus: a report of the first five years' work of the joint consultation clinic for neoplastic diseases of the Brompton Hospital and the Royal Cancer Hospital. *Thorax* 6, 1–16 (1951). In 7, (1).

BURDETTE, W. J. Effect of ligation of *Drosophila* larvae on tumor incidence. *Cancer Res.*, 14, 780–782 (1954). In 9, (470).

BURDETTE, W. J. Nucleotide levels in human cardiac muscle. *Am. Heart J.*, 54, 746–752 (1957). In 12, (709).

BUTLER, J. A. V. L'acide nucléique considéré comme un polyélectrolyte. *Colloq. int. Cent. nat. Rech. sci*, 57, 145–159 (1955). In 9, (482).

BUTLER, J A. V. The action of ionizing radiations on biological materials: facts and theories. *Radiat. Res.*, 4, 20–32 (1956). In 11, (502).

MAYNEORD, W. V. Radiological research. *Brit. J. Radiol.*, N.S., 27, 309–317 (1954). In 9, (153).

MAYNEORD, W. V. Some problems of radiation protection. *Brit. J. Radiol.*, N.S., 24. 525–537 (1951). In 7, (276).

MAYNEORD, W. V. The use of radioactive isotopes in diagnostic procedures. *Proceedings of the International Conference on the Peaceful Uses of Atomic Energy, Geneva*, 10, 209–217 (1955). (New York, United Nations, 1956). In 10, (235).

MAYNEORD, W. V., ANDERSON, W., EVANS, H. D., and ROSEN, D. Hydrogen peroxide yields in x-irradiated aqueous solutions: a sensitive method based on hydrazide chemiluminescence. *Radiat. Res.*, 3, 379–392 (1955). In 10, (250).

MAYNEORD, W. V., and BELCHER, E. H. Scintillation counting of gamma-rays. *Nature, Lond.*, 165, 930–931 (1950). In 7, (289). .

MAYNEORD, W. V., EVANS, H. D., and NEWBERY, S. P. An instrument for the formation of visual images of ionizing radiations. *J. sci. Instrum.*, 32, 45–50 (1955). In 10, (244).

MAYNEORD, W. V., and IRELAND, H. J. D. Radiography with thulium 170 and xenon 133. *Brit. J. Radiol.*, N.S., 29, 277–285 (1956). In 11, (108).

MAYNEORD, W. V., and NEWBERY, S. P. An automatic method of studying the distribution of activity in a source of ionizing radiation. *Brit. J. Radiol.*, N.S., 25, 589–596 (1952). In 8, (132).

MAYNEORD, W. V., TURNER, R. C., NEWBERY, S. P., and HODT, H. J. A method of making visible the distribution of activity in a source of ionizing radiation. *Nature, Lond.*, 168, 762–765 (1951). In 7, (292).

MERCER, E. H. Electron micrographs of cross-sections of protein monolayers. *Nature, Lond.*, 180, 87 (1957). In 12, (605).

MERCER, E. H. The fine structure of keratin. *Text. Res. J.*, 27, 860–866 (1957). In 12, (606)

MILL, W. A. Cancer of the larynx: laryngectomy after radiotherapy. *Proc. R. Soc. Med.*, 49, 73–84 (1956). In 11, (33).

ORR, S. F. D. An infra-⸗⸗⸗ ⸗⸗⸗⸗⸗⸗⸗ of steric hindrance in some *trans*-stilbene derivatives. *Spect⸗* · · ·. · · · · In 11, (896).

ORR, S. F. D. Infra-rec · · · polysaccharides. *Biochim. biophys. Acta* 14, 173–181 (1954). In 9, (896).

ORR, S. F. D., SIMS, P., and MANSON, D. The oxidation of 2-naphthylamine with benzoyl peroxide. *J. chem. Soc.*, 1337–1344 (1956). In 11, (907).

OSDENE, T. S, and TIMMIS, G. M. New ring systems of potential carcinogenic activity. 1. Derivatives of 4:9:10-triaza-1:2-5:6- and -1:2-7:8-dibenzanthracene. *J. chem. Soc.*, 4349–4354 (1955). In 10, (463).

OSDENE, T. S., and TIMMIS, G. M. A new synthesis of polycyclic compounds containing a fused isoquinoline ring system *Chem. & Ind (Rev.)*, 404 (1954). In 9, (363).

OSDENE, T. S., and TIMMIS, G. M. The reaction of cyanacetic acid and amide with o-nitroso amino compounds. *Chem. & Ind. (Rev.)*, 405–406 (1954). In 9, (364).

OSDENE, T. S., and TIMMIS, G. M. The synthesis of compounds with potential anti-folic acid activity. 3. 3.6-diaminoquinoxaline and derived compounds. *J. chem. Soc.*, 2027–2031 (1955). In 10, (445).

OSDENE, T. S., and TIMMIS, G. M. The synthesis of compounds with potential anti-folic acid activity. 4. 3:6-diaminopyrido(2:3)pyrazines. *J. chem. Soc.*, 2032–2035 (1955). In 10, (450).

OSDENE, T. S., and TIMMIS, G. M. The synthesis of compounds with potential anti-folic acid activity. 5. 4:7-diamino- and 2.4:7-trianimo-pteridine and its derivatives. *J. chem. Soc.*, 2036–2038 (1955). In 10, (454).

OSDENE, T. S., and TIMMIS, G. M. The synthesis of compounds with potential anti-folic activity. 6. Polyaza-1·2-benzanthracene derivatives. *J. chem. Soc.*, 2214–2218 (1955). In 10, (458).

OSDENE, T. S., and TIMMIS, G. M. The synthesis of 2.4-diamino-7-hydroxypteridine and its 6-carboxylic acid. *J chem. Soc.*, 2038–2039 (1955). In 10, (457).

PAIN, R. H., and BUTLER, J. A. V. The preparation and properties of ribonucleic acids from rat liver. *Biochem. J.*, 66, 299–302 (1957). In 12, (746).

PHILLIPS, D. M. P. The N-terminal groups of calf-thymus histones. *Biochem. J.*, 68, 35–40 (1957). In 12, (775).

PHILLIPS, D. M. P. Phosphoserine from the proteins of normal rabbit liver. *Biochim. biophys. Acta*, 21, 181–182 (1956). In 11, (577).

PRESS, E. M., and BUTLER, J. A. V. The action of ionizing radiations and of radiomimetic substances on deoxyribonucleic acid. 4. The products of the action of di-(2-chloroethyl) methylamine. *J. chem. Soc.*, 626–631 (1952) In 8, (780).

BUTLER, J. A. V. Effects of ultra-violet light on nucleic acid and nucleoproteins and other biological systems. *Proceedings of the 1st International Photobiological Congress, Amsterdam,* 1954. 111–117 (Wageningen, Veeman & Zonen, 1956). In 7, (475).

BUTLER, J. A. V. Effects of ultra-violet light on nucleic acid and nucleoproteins and other biological systems. *Experientia*, 11, 289–293 (1955). In 10, (529).

BUTLER, J. A. V. Effects of x-rays and radiomimetic agents on nucleic acids and nucleoproteins *Ciba Foundation Symposium on Ionizing Radiations and Cell Metabolism.* 59–69 (London Churchill, 1956). In 11, (491).

BUTLER, J. A. V. Nature of nucleotoxic substances. *Nature, Lond.,* 166, 18–19 (1950) In 7, (430).

BUTLER, J. A. V., and CONWAY, B. E. The action of ionizing radiations and of radiomimetic substances on deoxyribonucleic acid. 2 The effect of oxygen on the degradation of the nucleic acid by x-rays. *J. chem. Soc.,* 3418–3421 (1950). In 7, (435).

BUTLER, J. A. V., and CONWAY, B. E. The action of photochemically generated radicals from hydrogen peroxide on deoxyribonucleic acid and simple model substances *Proc. roy. Soc. B,* 141, 562–580 (1953) In 8, (446).

BUTLER, J. A. V., and CONWAY, B. E. Effects of salts on interactions in polyelectrolyte solutions *Nature, Lond.,* 172, 153–154 (1953). In 8, (465).

BUTLER, J. A. V., CONWAY, B. E., and JAMES, D. W. F. The effect of salts on polyelectrolyte interactions, with special reference to deoxyribonucleic acid *Trans. Faraday Soc.* 50, 612–623 (1954). In 9, (500).

BUTLER, J. A. V., and DAVISON, P. F. Deoxyribonucleoprotein, a genetic material *Advanc. Enzymol.* 18, 161–190 (1957). In 12, (716).

BUTLER, J. A. V., DAVISON, P.-F., JAMES, D. W. F., and SHOOTER, K. V. The histones of calf thymus deoxyribonucleoprotein. 1. Preparation and homogeneity. *Biochim. biophys. acta,* 13, 224–232 (1954). In 9, (577).

BUTLER, J. A. V., GILBERT, L., and JAMES, D. W. F. The action of ionizing radiations and of radiomimetic substances on deoxyribonucleic acid. 6. Physicochemical measurements of the action of bischloroethylmethylamine. *J. chem. Soc.,* 3268–3273 (1952). In 8, (467).

BUTLER, J. A. V., GILBERT, L., JAMES, D. W. F., and ROSS, W. C. J. Degradation of deoxyribonucleic acid by a "nitrogen mustard". *Nature, Lond.,* 168, 985–986 (1951). In 7, (439).

BUTLER, J. A. V., GILBERT, L. A., and SMITH, K. A. Radiomimetic action of sulphur and nitrogen "mustards" on deoxyribonucleic acid. *Nature, Lond.,* 165, 714–716 (1950). In 7, (443).

BUTLER, J. A. V., and JAMES, D. W. F. Gel-like behaviour of deoxyribonucleic acid solutions *Nature, Lond.,* 167, 844–845 (1951). In 7, (449).

BUTLER, J. A. V., JOHNS, E. W., LUCY, J. A., and SIMSON, P. The composition of nucleic acids prepared from rat and mouse tumours. *Brit. J. Cancer* 10, 202–208 (1956). In 11, (515).

BUTLER, J. A. V., LAURENCE, D. J. R., ROBINS, A. B., and SHOOTER, K. V. Molecular weights and physical properties of deoxyribonucleic acid. *Nature, Lond.,* 180, 1340 (1957). In 12, (821).

BUTLER, J. A. V., PHILLIPS, D. M., and SHOOTER, K. V. The influence of protein on heterogeneity of DNA. *Arch. Biochem.,* 71, 423–429 (1957). In 12, (803).

BUTLER, J. A. V., ROBINS, A. B., and SHOOTER, K. V. The viscous behaviour of dilute solutions of a strong polyelectrolyte (polystyrene sulphonate). *Proc. roy. Soc. A,* 241, 299–310 (1957). In 12, (781).

BUTLER, J. A. V., and SIMSON, P. After-effects of irradiation of DNA. *Proceedings of the 2nd Radiobiology Symposium, Liège,* 1954. 46–48 (London, Butterworth, 1955). In 9, (497).

BUTLER, J. A. V., and SMITH, K. A. The action of ionizing radiations and of radiomimetic substances on deoxyribonucleic acid. 1. The action of some compounds of the "mustard" type. *J. chem. Soc.,* 3411–3418 (1950). In 7, (453).

BUTLER, J. A. V., and SMITH, K. A. Degradation of deoxyribonucleic acid by free radicals. *Nature, Lond.,* 165, 847–848 (1950). In 7, (461).

BUTLER, L. O., JAMES, D. W. F., and TREHERNE, J. D. A compact and inexpensive Tiselius electrophoresis apparatus. *Clin. chim. Acta,* 1, 179–186 (1957). In 12, (860).

CARR, J. G., and HARRIS, R. J. C. Preservation of the agent of the Rous sarcoma No.1 by freeze-drying. *Brit. J. Cancer,* 5, 95–105 (1951). In 7, (476).

CARR, J. G., and HARRIS, R. J. C. The preparation and assay of the Rous No.1 sarcoma agent. *Brit. J. Cancer,* 5, 83–94 (1951). In 7, (464).

CASE, R. A. M. Cohort analysis of cancer mortality in England and Wales, 1911–1954 by site and sex. *Brit. J. soc. Med.,* 10, 172–199 (1956). In 11, (592).

CASE, R. A. M. Cohort analysis of mortality rates as an historical or narrative technique. *Brit. J. soc. Med.,* 10, 159–171 (1956). In 11, (579).

PURDOM, C. E. Autonomous action of lethal mutations induced in the germ cells of *Drosophila melanogaster* by 2-chloroethyl methanesulphonate. *Nature, Lond.*, 180, 81–83 (1957). In 12, (902).

RAVEN, R. W. Cancer of the breast treated by oophorectomy. *Brit. med. J.*, i, 1343–1345 (1950). In 7, (101).

RAVEN, R. W. Combined cervico-buccal partial pharyngectomy for cancer of the oropharynx. *Brit. J. Surg.*, 39, 503–505 (1952). In 8, (54).

RAVEN, R. W. Liver surgery in relation to diseases of the colon and rectum. *Proc. R. Soc. Med.*, 50, 775–786 (1957). In 12, (140).

RAVEN, R. W. The properties and surgical problems of malignant melanoma. *Ann. R. Coll Surg. Engl.*, 6, 28–55 (1950). In 7, (109).

RAVEN, R. W. Smoking habits of schoolboys. *Lancet*, i, 1139–1141 (1957). In 12, (138).

RAVEN, R. W. The surgical treatment of carcinoma of the pharynx. *Proc. R. Soc. Med.*, 46, 770–776 (1953). In 8, (57).

RAVEN, R. W., and CHRISTIE, A. C. Haemangiosarcoma a case with lymphatic and haematogenous metastases. *Brit. J. Surg.*, 41, 483–489 (1954) In 9, (1).

REID, E. Carcinogenic action of 2'-chloro-4-dimethylaminostilbene, in relation to (A) possible effect of growth hormone, and (B) composition of liver cytoplasm. *Brit. J Cancer*, 10, 123–128 (1956). In 11, (461).

REID, E. Effect of growth hormone on liver proteins and nucleic acids *The hypophyseal growth hormone, nature and actions.* Ed. by Smith, Gaebler and Long. 235–246 (New York, McGraw-Hill, 1955). In 10, (514)

REID, E. Growth hormone and adrenocortical hormones in relation to experimental tumours: a review. *Cancer Res.*, 14, 249–266 (1954) In 9, (452).

REID, E. Hormonal effects on liver and kidney cytoplasm. *J. Endocrin.*, 13, 319–329 (1956). In 11, (448).

REID, E. Pituitary and adrenal influences on the composition of liver cytoplasm. *Nature, Lond.*, 175, 461–463 (1955). In 10, (536).

REID, E., and LEWIN, I Adenosine deaminase nucleoside phosphorylase and xanthine oxidase in liver tumours. *Brit. J. Cancer*, 11, 494–498 (1957). In 12, (691).

REID, E., and O'NEAL, M. A. "Free" enzymic activity of mitochondrial fractions from liver tumours *Brit. J. Cancer*, 10, 587–593 (1956). In 11, (467)

REID, E., O'NEAL, M. A., and LEWIN, I. Hormones and liver cytoplasm. 2. Adenosine triphosphatase, glucose 6-phosphatase and xanthine oxidase as affected by hypophysectomy, growth-hormone treatment and adrenalectomy. *Biochem. J.*, 64, 730–734 (1956). In 11, (480)

REID, E., O'NEAL, M. A., STEVENS, B. M., and BURNOP, V. C. E. Hormonal influences on the incorporation of injected precursors into the protein and ribonucleic acid of liver cytoplasm. *Biochem. J.*, 64, 33–38 (1956). In 11, (474).

REID, E., and STEVENS, B. M. Hormones and liver cytoplasm. 4. Ribonucleotides, ribonucleic acid synthesis and protein synthesis after adrenalectomy. *Biochem. J.*, 67, 262–274 (1957). In 12, (696).

REVELL, S. H. Chromosome breakage by x-rays and radiomimetic substances in *Vicia.* *Heredity* 6, *suppl.*, 107–124 (1953) In 8, (786).

REVELL, S. H. A new hypothesis for "chromatid" changes. *Proceedings of 2nd Radiobiology Symposium, Liège.* 1954. 243–253 (London, Butterworth, 1955). In 9, (858).

RIGBY-JONES, P. The influence of various factors on metastases in carcinoma of the breast. *Brit. J. Cancer*, 7, 431–437 (1953). In 8, (224).

ROBERTS, J. J., and ROSS, W. C. J. Aryl-2-halogenoalkylamines. 10. The preparation of some derivatives of 1-aryl-3:4-dihalogenobutanes. *J. chem. Soc.*, 4288–4295 (1952). In 8, (805).

ROBERTS, J. J., and ROSS, W. C. J. Sulphonium compounds related to aryldi-2-halogenoalkylamines. *J. chem. Soc.*, 1388–1389 (1955). In 10, (437).

ROBERTS, J. J., and WARWICK, G. P. Mode of action of alkylating agents: formation of S-ethyl-cysteine from ethyl methanesulphonate *in vivo.* *Nature, Lond.*, 179, 1181–1182 (1957). In 12, (480).

ROE, E. M. F. Applications of the reflecting microscope. *Research, Lond.*, 8, 147–157 (1955). In 10, (798).

ROE, E. M. F. Some effects of environment on electronic spectra. *Colloquium Spectroscopicum Internationale, Amsterdam,* 1956. 515–533 (London, Pergamon, 1957). In 12, (1026).

ROSEN, D., and BOMAN, H. G. Effects of gamma rays on solutions of human serum albumin. 2. Chromatographic studies. *Arch. Biochem.*, 70, 277–282 (1957). In 12, (392).

CASE, R. A. M. The expected frequency of bladder tumour in works populations. *Brit. J. industr. Med.*, **10**, 114–120 (1953). In 8, (473).

CASE, R. A. M. Incidence of death from tumours of the urinary bladder. *Brit. J. soc. Med*, **7**, 14–19 (1953). In 8, (480).

CASE, R. A. M., and HOSKER, M. E. Tumour of the urinary bladder as an occupational disease in the rubber industry in England and Wales. *Brit. J., soc. Med.*, **8**, 39–50 (1954). In 9, (605).

CASE, R. A. M., HOSKER, M. E., McDONALD, D. B., and PEARSON, J. T. Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. 1. The role of aniline, benzidine, alpha-naphthylamine and·beta-naphthylamine. *Brit. J. industr. Med.*, **11**, 75–104 (1954). In 9, (617).

CASE, R. A. M., and LEA, A. J. Mustard gas poisoning, chronic bronchitis, and lung cancer: an investigation into the possibility that poisoning by mustard gas in the 1914–1918 war might be a factor in the production of neoplasia. *Brit. J. soc. Med.*, **9**, 62–72 (1955). In 10, (554).

CASE, R. A. M., and PEARSON, J. T. Tumours in the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. *Brit. J. industr. Med.*, **11**, 213–216 (1954). In 9, (647).

CHARLES, R. T. The recording of inbred strains of mice used in cancer research. *J. Anim. Tech. Ass.*, **7**, 22–25 (1936). In 11, (625).

CHESTER, A. M., and LEWIS, C. B. Anaesthesia for total laryngectomy. *Anaesthesia*, **5**, 21–25 (1950). In 7, (17).

CHRISTIE, A. C. Duodenal carcinoma with neoplastic transformation of the underlying Brunner's glands. *Brit. J. Cancer*, **7**, 65–67 (1953). In 8, (6).

CHRISTIE, A. C. Ossification in intestinal neoplasms. a report of three cases. *J. Path. Bact.*, **63**, 338–340 (1951). In 7, (22).

CLAIN, A., and HUNT, A. H. Adrenalectomy for intracranial metastases from carcinoma of the breast. *Brit. med. J.*, **ii**, 627–629. In 11, (5).

COFFIN, F. The restoration of function after large operations about the face and jaws. *J. Laryng. Otol.*, **67**, 177–188 (1953). In 8, (13).

COHN. P. The distribution of radioactivity in tissues of the rat following the administration of a nitrogen mustard derivative. *Brit. J. Cancer* **11**, 258–267 (1957). In 12, (793).

COHN, P.. and BUTLER, J. A. V. Fractionation of microsomal proteins by a non-ionic detergent. *Biochim. biophys. Acta.*, **25**, 222–223 (1957). In 12, (810).

CONWAY, B E. The "after-effect" of irradiation of deoxyribonucleic acid in oxygenated solutions. *Brit. J. Radiol.*, N.S., **27**, 42–47 (1954). In 9, (596).

CONWAY, B. E. Effect of urea on the viscosity of deoxyribonucleic acid solutions. *J. Polym. Sci.*, **20**, 497–502 (1956). In 11, (554).

CONWAY, B. E. Effects of salts on the viscosity of polyelectrolyte solutions. *J. Polym. Sci.*, **18**, 257–274 (1955). In 10, (534).

CONWAY, B E., and BUTLER, J A. V. The action of denaturing agents on deoxyribonucleic acid. *J. chem. Soc*, 3075–3082 (1952). In 8, (486).

CONWAY, B. E., and BUTLER, J. A. V. The action of ionizing radiations and of radiomimetic substances on deoxyribonucleic acid. 5. Some experiments on the action of x-rays and free radicals. *J. chem. Soc.*, 834–838 (1952). In 8, (494).

CONWAY, B E., and BUTLER, J. A. V. Effect of salts on the interaction of nucleic acid particles. *J. Polym. Sci.*, **12**, 199–207 (1954). In 9, (512).

CONWAY, B. E., GILBERT, L., and BUTLER, J. A. V. The action of ionizing radiations and of radiomimetic substances on deoxyribonucleic acid. 3. The molecular weights of deoxyribonucleic acid degraded by x rays and by treatment with a "nitrogen mustard". *J. chem. Soc.*, 3421–3425 (1950). In 7, (487).

COPP, F. C., and TIMMIS, G. M. Potential tumour inhibitors. Esters of 4-di-(2-hydroxyethyl) aminopyridine and quaternary derivatives of 2:3-dihydroglyoxalino [1:2-a] -pyridine. *J. chem. Soc.*, 2021–2027 (1955). In 10, (439).

CRATHORN, A. R., and HUNTER, G. D. The effect of nitrogen m   .... on of amino acids into protein by *Staphylococcus aureus. Biochem.*   '   · .'

DALLEY, V. M. Cancer of the middle ear. *J. Fac. Radiol., Lond.*, 4,

DAOUST, R. Localization of deoxyribonuclease in tissue sections: a new approach to the histochemistry of enzymes. *Exp. Cell Res.*, **12**, 203–211 (1957). In 12, (868).

DARCY   .... demonstration of a substance in rat blood associated with tissue
                                          957) In 12, (877).

DARCY,   etween the blood of normal and of tumour-bearing
     rats. *Nature, Lond.*, **176**, 643–644 (1955). In 10, (581).

Rosen, D., Brohult, S., and Alexander, P. Effects of gamma rays on solutions of human serum albumin. 1. Sedimentation studies. *Arch. Biochem.*, 70, 266–276 (1957). In 12, (381).

Ross, W. C. J. Aryl-2-halogenoalkylamines. 6. Reaction with certain organic sulphur compounds. *J. chem. Soc.*, 815–818 (1950). In 7, (622).

Ross, W. C. J. Biological action of x-rays, nitrogen mustards, diepoxides and peroxides. *Nature, Lond.*, 165, 808–809 (1950). In 7, (626).

Ross, W. C. J. The correlation of chemical structure with cytotoxic activity in the nitrogen mustards. *Acta Un. int. Cancr.*, 10, 159–160 (1954). In 9, (337).

Ross, W. C. J. The reactions of certain epoxides in aqueous solutions. *J. chem. Soc.*, 2257–2271 (1950). In 7, (629).

Ross, W. C. J., and Davis, W. Some potentially cytotoxic alkylsulphonates. *J. chem. Soc.*, 2420–2422 (1957). In 12, (467).

Ross, W. C. J., and Warwick, G. P. Aryl-2-halogenoalkylamines. 16. The preparation of derivatives of 4-[di-(2-chloroalkyl)amino]azobenzenes. *J. chem. Soc.*, 1364–1369 (1956). In 11, (354).

Ross, W. C. J., and Warwick, G. P. Aryl-2-alogenoalkylamines. 17. The ultra-violet absorption spectra of some 4-di-(2-chloroethyl)aminoazobenzenes in neutral and acidic ethanol solutions. *J. chem. Soc.*, 1719–1724 (1956). In 11, (359).

Ross, W. C. J., and Warwick, G. P. Aryl-2-halogenoalkylamines. 18. The rates of reduction of substituted 4-di-(2-chloroethyl)aminoazoxybenzenes by stannous chloride, hydrazine, and the xanthine oxidase-xanthine system. *J. chem. Soc.*, 1724–1732 (1956). In 11, (365).

Ross, W. C. J., and Warwick, G. P. Reduction of cytotoxic azo compounds by hydrazine and by the xanthine oxidase-xanthine system. *Nature, Lond.*, 176, 298–299 (1955). In 10, (477).

Ross, W. C. J., and Warwick, G. P. The spectra of o-carboxyazobenzene derivatives. *Chem.: & Ind. (Rev.)*, 745–747 (1957). In 10, (479).

Ross, W. C. J., Warwick, G. P., and Roberts, J. J. Aryl-2-halogenoalkylamines. 14. Some compounds possessing latent cytotoxic activity. *J. chem. Soc.*, 3110–3116 (1955). In 10, (425).

Shooter, K. V. The physical chemistry of deoxyribosenucleic acid. *Progr. Biophys.*, 8, 310–346 (1957). In 12, (822).

Shooter, K. V. Physical state of deoxyribonucleic acid in rat thymus homogenates. *Nature, Lond.*, 173, 824–825 (1954). In 9, (604).

Shooter, K. V., and Butler, J. A. V. Physical fractionation of DNA by centrifugation and other processes. *J. Polym. Sci.*, 23, 705–711 (1957). In 12, (812).

Shooter, K. V., and Butler, J. A. V. Apparent heterogeneity of deoxyribonucleic acid: sedimentation experiments at low concentrations. *Nature, Lond.*, 175, 500–502 (1955). In 10, (552).

Shooter, K. V., and Butler, J. A. V. Fractionation of deoxyribonucleic acid by physical procedures. *Nature, Lond.*, 177, 1033–1034 (1956). In 11, (546).

Shooter, K. V., and Butler, J. A. V. Sedimentation of deoxyribonucleic acid at low concentrations. *Trans. Faraday Soc.*, 52, 734–742 (1956). In 11, (528).

Shooter, K. V., Davison, P. F., and Butler, J. A. V. Physical properties of thymus nucleoprotein. *Biochim. biophys. Acta*, 13, 192–198 (1954). In 9, (570).

Shooter, K. V., Pain, R. H., and Butler, J. A. V. The effect of heat and x-rays on deoxyribonucleic acid. *Biochim. biophys. Acta*, 20, 497–502 (1956). In 11, (548).

Simonsen, M. Artificial production of immunological tolerance: induced tolerance to heterologous cells and induced susceptibility to virus. *Nature, Lond.*, 175, 763–764 (1955). In 10, (796).

Sinclair, W. K. Artificial radioactive sources for interstitial therapy. *Brit. J. Radiol., N.S.*, 25, 417–419 (1952). In 8, (140).

Sinclair, W. K. Comparison of Geiger-counter and ion-chamber methods of measuring gamma-radiation. *Nucleonics* 7, 21–26 (1950). In 7, (299).

Sinclair, W. K. Measurement and handling of radioactive materials for therapeutic use. *Nucleonics*, 9, 35–42 (305). In 7, (305).

Sinclair, W. K., Abbatt, J. D., Farran, H. E. A., Harriss, E. B., and Lamerton, L. F. A quantitative autoradiographic study of radioiodine distribution and dosage in human thyroid glands. *Brit. J. Radiol., N.S.*, 29, 36–41 In 11, (117).

Sinclair, W. K., and Blondal, H. P32 beta sources for superficial therapy. *Brit. J. Radiol., N.S.*, 25, 360–368 (1952). In 8, (143).

Sinclair, W. K., and Emery, E. W. The half-life of iodine-131 and the anomalous decay of exposed sodium iodide sources. *Brit. J. Radiol., N.S.*, 23, 576–578 (1950). In 7, (313).

DARCY, D. A. The reaction of the rabbit to frozen homografts. *J. Path. Bact.*, 70, 143–156 (1955). In 10, (565).

DARCY, D. A. Survival of adrenal gland homografts in the rabbit's skin. *Nature, Lond.*, 170, 805 (1952). In 8, (499).

DAVIS, W. The reaction of nitrogen mustards with molecules of biological importance. *Acta Un int. Cancr.*, 10, 110–111 (1954). In 9, (335).

DAVIS, W., EVERETT, J. L., and ROSS, W. C. J. Aryl-2-halogenoalkylamines. 7. Some derivatives of 2-naphthyldi-2'-halogenoalkylamines. *J. chem. Soc.*, 1331–1337 (1950). In 7, (492).

DAVIS, W., ROBERTS, J. J., and ROSS, W. C. J. Aryl-2-halogenoalkylamines. 13. Chloroethyl amino-derivatives of some phenoxyalkanoic acids and of some substituted amino acids. *J. chem. Soc.*, 890–895 (1955). In 10, (419).

DAVIS, W., and ROSS, W. C. J. A comparison of the solubilities of esters derived from some ... with those obtained from ... radiomimetic compounds. In 7, (499).

DAVIS, W., and Ross, W. C. J. ... the ... (506)

DAVIS, W., and ROSS, W. C. J. The isolation of β-sitosterol from Lithospermum officinale. *Chem. & Ind. (Rev.)*, 1739–1740 (1955). In 10, (476).

DAVIS, W., and ROSS, W. C. J. Aryl-2-halogenoalkylamines. 11. The reaction of NN-di-2-chloroethyl-2-naphthylamine, with some phosphoric acid derivatives. *J. chem. Soc.*, 4296–4300 (1952). In 8, (500).

DAVISON, P. F. Chromatography of histones. *Biochem. J.*, 66, 708–712 (1957). In 12, (765).

DAVISON, P. F. Histones from normal and malignant cells. *Biochem. J.*, 66, 703–707 (1957). In 12, (760).

DAVISON, P. F., and BUTLER, J. A. V. The chemical composition of calf thymus nucleoprotein. *Biochim. biophys. Acta*, 21, 568–573 (1956). In 11, (522).

DAVISON, P. F., CONWAY, B. E., and BUTLER, J. A. V. The nucleoprotein complex of the cell nucleus, and its reactions. *Progr Biophys.*, 4, 148–194 (1954). In 9, (521).

DAVISON, P. F., JAMES, D. W. F., SHOOTER, K. V., and BUTLER, J. A. V. The histones of calf thymus deoxyribonucleoprotein. 2. Electrophoretic and sedimentation behaviour and a partial fractionation. *Biochim. biophys. Acta* 15, 415–424 (1954). In 9, (586).

DAVISON, P. F., and M ... of cell nuclei isolated in aqueous media. *Exp. Cell Res.*, 11, ...

DAVISON, P. F., and SF ... trophoretic and chromatographic studies of whole and fractionated calf thymus histones. *Bull. Soc. chim. Belg.*, 65, 85–96 (1956). In 11, (561).

DEVIK, F., ELSON, L. A., KOLLER, P. C., and LAMERTON, L. F. The influence of diet on the Walker rat carcinoma 256, and its response to x-radiation — cytological and histological investigations. *Brit. J. Cancer*, 4, 298–314 (1950). In 7, (509).

DE WINTER, J. G. Some preliminary clinical observations on the use of radioactive isotopes for the localisation of brain tumours. *Brit. J. Radiol.*, N.S., 24, 280–284 (1951). In 7, (168).

EASTY, D. M., LEDOUX, L., and AMBROSE, E. J. The action of ribonuclease on neoplastic growth. 3. Studies by interference microscopy. *Biochim. biophys. Acta*, 20, 528–537 (1956). In 11, (295).

EASTY, G. C., and AMBROSE, E. J. The antigenic composition of mouse ascites tumour cells using *in vitro* and gel-diffusion techniques. *Brit. J. Cancer*, 11, 287–295 (1957). In 12, (427).

EASTY, G. C., and AMBROSE, E. J. An interferometric method of quantitative analysis of antigen-antibody reactions in gels. *J. exp. Biol.*, 34, 60–70 (1957). In 12, (414).

ELSON, L. A. The carcinogenic action of aminostilbene derivatives in rats maintained on high and low protein diets. *Brit. J. Cancer*, 6, 392–399 (1952). In 8, (505).

ELSON, L. A. A comparison of the effects of radiation and radiomimetic chemicals on the blood. *Brit. J. Haemat.*, 1, 104–116 (1955). In 10, (583).

ELSON, L. A. Comparison of the physiological response to radiation and to radiomimetic chemicals. *Proceedings of the 2nd Radiobiology Symposium, Liège, 1954.* 235–242 (London, Butterworth, 1955). In 9, (651).

ELSON, L. A., GALTON, D. A. G., LAMERTON, L. F., and TILL, M. Comparison of the physiological response to radiation and to radiomimetic chemicals — bone marrow effects. *Progress in Radiobiology: proceedings of the 4th international conference on radiobiology, Cambridge, 1955.* 285–288 (Edinburgh, Oliver & Boyd, 1956). In 10, (596).

SINCLAIR, W. K., and HOLLOWAY, A. F. Half-lives of some radioactive isotopes. *Nature, Lond.*, 167, 365 (1951). In 7, (316).

SINCLAIR, W. K., and NEWBERY, S. P. A direct reading meter for the measurement of highly active samples of gamma-emitting radioisotopes. *J. sci. Instrum.*, 28, 234–235 (1951). In 7, (318).

SINCLAIR, W. K., and TROTT, N. G. The construction and measurement of beta-ray applicators for use in ophthalmology. *Brit. J. Radiol., N.S.*, 29, 15–23 (1956). In 11, (138).

SINCLAIR, W. K., TROTT, N. G., and BELCHER, E. H. The measurement of radioactive samples for clinical use. *Brit. J. Radiol., N.S.*, 27, 565–574. In 9, (162).

SMITH, C. The introduction and breeding of the Chinese striped hamster (Cricetulus griseus) in Great Britain. *J. Anim. Tech. Ass.*, 7, 59–60. In 12, (1050).

SMITHERS, D. W. Adenocarcinoma of the oesophagus. *Thorax*, 11, 257–267 (1956). In 11, (216).

SMITHERS, D. W. Applications of radiobiology to radiation therapy. *Proceedings of the 2nd National Cancer Conference, Cincinnatti, 1952.* 1015–1027 (New York, American Cancer Society, 1954). In 9, (89).

SMITHERS, D. W. The association of cancer of the gastric cardia with partial thoracic stomach, short oesophagus and peptic ulceration. *Brit. J. Radiol., N.S.*, 23, 261–269 (1950). In 7, (137).

SMITHERS, D. W. The association of cancer of the stomach and oesophagus with herniation at the oesophageal hiatus of the diaphragm. *Brit. J. Radiol., N.S.*, 28, 554–564 (1955). In 10, (129).

SMITHERS, D. W. Cancer of the breast and the menopause. *J. Fac. Radiol., Lond.*, 4, 90–95 (1952). In 8, (231).

SMITHERS, D. W. Carcinoma of the bronchus: a radiotherapy viewpoint *J. Fac. Radiol., Lond.*, 6, 174–181 (1955). In 10, (140).

SMITHERS, D. W. Clinical cancer research. *Lancet*, i, 253–257 (1956). In 11, (234)

SMITHERS, D. W. Clinical experience with isotopes in Europe. *Proceedings of the 2nd National Cancer Conference, Cincinnatti, 1952.* 1675–1684 (New York, American Cancer Society, 1954). In 9, (101).

SMITHERS, D. W. Facts and fancies about cancer of the lung. *Brit. med. J.*, i, 1235–1239 (1953). In 8, (246).

SMITHERS, D. W. Progress in the treatment of breast cancer. *Lancet*, ii, 495–501 (1952). In 8, (239).

SMITHERS, D. W. Radioactive isotopes in medicine. *Proc. R. Soc. Med.*, 48, 944–946 (1954). In 10, (148).

SMITHERS, D. W. Records and presentation of results. *British Practice in Radiotherapy.* Ed. by Carling, Windeyer and Smithers. 501–516 (London, Butterworth, 1955. In 10, (187).

SMITHERS, D. W. Rotation therapy. *J. Fac. Radiol., Lond.*, 6, 73–83 (1954). In 9, (68).

SMITHERS, D. W. Some varied applications of radioactive isotopes to the localisation and treatment of tumours. *Acta radiol., Stockh.*, 35, 49–61 (1951). In 7, (203).

SMITHERS, D. W. The treatment of carcinoma of the oesophagus. *Ann. R. Coll. Surg. Engl.*, 20, 36–49 (1957). In 12, (152).

SMITHERS, D. W. Die Zukunft der Bewegungsbestrahlung: Rotationsbestrahlung bei Patienten mit tief gelegenen Tumoren. *Strahlentherapie*, 95, 79–88 (1954). In 9, (79).

SMITHERS, D. W., and GORDON, H. Cancer of the skin. *British Practice in Radiotherapy.* Ed. by Carling, Windeyer and Smithers. 391–406 (London, Butterworth, 1955). In 10, (171).

SMITHERS, D. W., and PAYNE, P. M. Some graphical methods of presenting results of cancer treatment. *Brit. J. Radiol., N.S.*, 29, 65–75 (1956). In 11, (239).

SOLOMON, J. B. Glutamic dehydrogenase in the developing chick embryo. *Biochem. J.*, 66, 264–270 (1957). In 12, (1052).

SOLOMON, J. B. Increase of deoxyribonucleic acid and cell number during morphogenesis of the early chick embryo. *Biochim. biophys. Acta*, 23, 24–27 (1957). In 12, (1059).

SOLOMON, J. B. Nucleic acid content of the egg of the domestic fowl. *Biochim. biophys. Acta*, 23, 211–212 (1957). In 12, (1063).

SOLOMON, J. B. Nucleic acid content of early chick embryos and the hen's egg. *Biochim. biophys. Acta*, 24, 584–591 (1957). In 12, (1065).

SOLOMON, J. B. Synthesis of nucleic acids by chick blastoderms grown on synthetic medium. *Biochim. biophys. Acta*, 25, 69–73 (1957). In 12, (1073).

SPICKETT, R. G. W., and TIMMIS, G. M. Quaternary salts from 2-chloro-5-nitropyrimidines. Part 1. Preparations and some reactions. *J. chem. Soc.*, 4354–4359 (1955). In 10, (468).

SPICKETT, R. G. W., and TIMMIS, G. M. The synthesis of compounds with potential anti-folic acid activity. Part 1. 7-amino- and 7-hydroxy-pteridines. *J. chem. Soc.*, 2887–2895 (1954). In 9, (345).

EVANS, H. D. Automatic scanning methods for plotting the distribution of radioactive isotopes in the human body. *Sci. J. R. Coll. Sci.*, 24, 106–119 (1954). In 9, (126).

EVERETT, J. L., and KON, G. A. R. The preparation of some cytotoxic epoxides. *J. chem. Soc.*, 3131–3135 (1950). In 7, (533).

EVERETT, J. L., ROBERTS, J. J., and ROSS, W. C. J. Aryl-2-halogenoalkylamines. 12. Some carboxylic derivatives of NN-di-2-chloroethylaniline. *J. chem. Soc.*, 2386–2392 (1953). In 8, (514).

FAHMY, O. G., and BIRD, M. J. Chromosome breaks among recessive lethals induced by chemical mutagens in *Drosophila mel* . . .

FAHMY, O. G., and FAHMY, M. J. . . . . *Progress in Radiobiology:* . . . . Cambridge, 1955. 205–208 (Edinburgh, Oliver & Boyd, 1956). In 10, (041).

FAHMY, O. G., and FAHMY, M. J. Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. 2. The mechanism of induction of dominant lethals by 2:4:6-tri(ethyleneimino)-1:3:5-triazine. *J. Genet.*, 52, 603–619 (1954). In 9, (659).

FAHMY, O. G., and FAHMY, M. J. . . . . of carcinogens and tumour inhibitors in *Drosophila* . . . . tural changes induced by 2:4:6-tri-(ethyleneimino)-1 . . . . (1955). In 10, (600).

FAHMY, O. G., and FAHMY, M. J. Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. 4. The cell stage during spermatogenesis and the induction of intra- and inter-genic mutations by 2:4:6-tri (ethyleneimino) 1:3:5-triazine. *J. Genet.*, 53, 563–584 (1955). In 10, (619).

FAHMY, O. G., and FAHMY, M. . . . . ction of carcinogens and tumour inhibitors in *Drosophila* . . . . netic response to the alkylating mutagens and x-radiation . . . . In 11, (630).

FAHMY, O. G., and FAHMY, M. J. Further evidence for differential effects of mutagens in *Drosophila melanogaster*. *J. Genet.*, 55, 280–287 (1957). In 12, (890).

FAHMY, O. G., and FAHMY, M. J. Mutagenic response to the alkyl-methane-sulphonates during spermatogenesis in *Drosophila melanogaster*. *Nature, Lond.*, 180, 31–34 (1957). In 12, (889).

FAHMY, O. G., and FAHMY, M. J. Mutagenicity of 2-chloroethyl methanesulphonate in *Drosophila melanogaster*. *Nature, Lond.*, 177, 996–997 (1956). In 11, (629).

FELTON, D. G. I. Some reactions of ethyl cyanoacetate. *J. chem. Soc.*, 515–517 (1955). In 10, (410).

FELTON, D. G. I., and ORR, S. F. D. The infra-red spectra of some esters, nitriles, and ester-nitriles. *J. chem. Soc.*, 2170–2176 (1955). In 10, (413).

FELTON, D. G. I., OSDENE, T. S., and TIMMIS, G. M. The synthesis of compounds with potential anti-folic acid activity. 2. 5:7:9,10-tetra-aza-1:2-benzanthracene and related compounds. *J. chem. Soc.*, 2895–2904 (1954). In 9, (354).

FELTON, D. G. I., and TIMMIS, G. M. The reaction between NN-di-2'-chloroalkly-2-naphthyl-amines and 4-amino-5-nitrosopyrimidines. *J. chem. Soc.*, 2881–2886 (1954). In 9, (339).

FICARI, A. A case of lymphosarcoma with metastases in unusual situations. *J. Path. Bact.* 62, 103–111 (1950). In 7, (26).

FOULDS, L. Experimental and histological studies of the responsiveness of mammary tumours to hormones. *Ann. R. Coll. Surg. Engl.*, 18, 384–395 (1956). In 11, (752).

FOULDS, L. Experimental study of the course and regulation of tumour growth. *Ann. R. Coll. Surg., Engl.*, 9, 93–101 (1951). In 7, (538).

FOULDS, L. The experimental study of tumor progression: a review. *Cancer Res.*, 14, 327–339 (1954). In 9, (676).

FOULDS, L. The histologic analysis of mammary tumors of mice. *J. nat. Cancer Inst.*, 17, 701–794 (1956). In 11, (649).

FOULDS, L. Tumor progression. *Cancer Res.*, 17, 355–356 (1957). In 12, (904).

FOURACRES, F. A., and WHITTICK, J. W. The relationship of molluscum sebaceum (kerato-acanthoma) to spontaneously healing epithelioma of the skin. *Brit. J. Cancer* 7, 58–64 (1953). In 8, (25).

FOX, M., and ALEXANDER, P. Post-effects dans la polymérisation des solutions aqueuses d'acide méthacrylique amorcée par les rayons x. *J. Chim. phys*, 52, 710–717 (1955). In 10, (324).

GALLUZZI, S., and PAYNE, P. M. Brain metastases from primary bronchial carcinoma: a statistical study of 741 necropsies. *Brit. J. Cancer*, 10, 408–414 (1956). In 11, (150).

GALLUZZI, S., and PAYNE, P. M. Bronchial carcinoma: a statistical study of 741 necropsies with special reference to the distribution of blood-borne metastases. *Brit. J. Cancer*, 9, 511–527 (1955). In 10, (64).

SPIEGLER, G. Photographic protection measurements with x-rays, gamma and beta rays. *Photogr. J.,* 91B, 128–133 (1951). In 7, (320).

SPIEGLER, G., OLLERENSHAW, R., and MACEY, R. High contrast: a potential source of radiographic error. *Brit. J. Radiol., N.S.,* 30, 481–488 (1957). In 12, (268).

STACEY, K. A., and ALEXANDER, P. Aggregation and hydrogen '   yl alcohol. *Proceedings of the International Symposium on*   *'an and Turin,* 1954. 889–896 (Rome, Consiglio Nazionale de

STANTON, W. R., and SINCLAIR, W. K. Effect of high concentration of phosphorus-32 on growth of the potato. *Nature, Lond.,* 167, 234–235 (1951). In 7, (326).

STAPLETON, J. E., McKISSOCK, W., and FARRAN, H. E. A. The uptake of radioactive phosphorus in normal brain and brain tumours. *Brit. J. Radiol., N.S.,* 25, 69–75 (1952). In 8, (251).

STEVENS, B. M., and REID, E. Hormones and liver cytoplasm. 3. Succinic dehydrogenase, nucleases and 'polymerized' ribonucleic acid as affected by hypophysectomy, growth-hormone treatment and adrenalectomy. *Biochem. J.,* 64, 735–740 (1956). In 11, (485).

STEVENSON, J. J. Horizontal body section radiography. *Brit. J. Radiol., N.S.,* 23, 319–334 (1950). In 7, (146).

TIMMIS, G. M. Antifolic acids and antipurines in chemotherapy. *J. Pharm., Lond.,* 9, 81–91 (1957). In 12, (504).

TIMMIS, G. M., COOKE, I., and SPICKETT, R. G. W. Synthetic chemical investigations related to the metabolism of purines. *Ciba Foundation Symposium on the Chemistry and Biology of Purines.* 134–142 (London, Churchill, 1957). In 12, (525).

TIMMIS, G. M., and FELTON, D. G. I., COLLIER, H. O. J., and HUSKINSON, P. L. Structure-activity relations in two new series of anti-folic acids. *J. Pharm., Lond.,* 9, 46–67 (1957). In 12, (482).

TIMMIS, G. M., FELTON, D. G. I., and OSDENE, T. S. The use of o-aminonitrose compounds in the synthesis of pteridines and some analogous ring systems. *Ciba Foundation Symposium on Chemistry and Biology of Pteridines.* 93–103. (London, Churchill, 1954). In 9, (365).

TROTT, N. G., and WHEATLEY, B. M. Tantulum 182 wire gamma-ray applicators for use in ophthalmology. *Brit. J. Radiol., N.S.,* 29, 13–14 (1956). In 11, (136).

TURNER, R. C., and NEWBERY, S. P. A probe type direct reading dosage-rate meter. *Brit. J. Radiol., N.S.,* 23, 424–429 (1950). In 7, (330).

VAN PATTER, H. T., and WHITTICK, J. W. Heterotopic ossification in intestinal neoplasms. *Amer. J. Path.,* 31, 73–91 (1955). In 10, (41).

VASSAR, P. S., and TAYLOR, D. M. Effects of hypoxia on iron absorption in rats. *Proc. Soc. exp. Biol., N.Y.,* 93, 504–506 (1956). In 11, (250).

WALLACE, D. M. Bladder tumours. *Recent advances in urology.* Ed. Hanley. 148–175 (London, Churchill, 1957). In 12, (166).

WALLACE, D. M. The ill-effects of radiotherapy. *Brit. J. Urol.,* 26, 364–368 (1954). In 9, (8).

WALLACE, D. M., STAPLETON, J. E., and TURNER, R. C. Radioactive tantalum wire implantation as a method of treatment for early carcinoma of the bladder. *Brit. J. Radiol., N.S.,* 25, 421–424 (1952). In 9, (274).

WALTON, R. J., and SINCLAIR, W. K. Intracavitary irradiation with radioactive colloidal gold in the palliative treatment of malignant pleural and peritoneal effusions. *Brit. med. Bull.,* 8, 165–172 (1952) In 8, (266).

            ions (24 Na and 82 Br) in the treatment

        ed kidney

carcinoma: complement fixation reactions with cytoplasmic par...   *'ancer,* 10, 553–559 (1956). In 11, (915).

WEILER, E. Antigenic differences between normal hamster kidney and stilboestrol induced kidney carcinoma: histological demonstration by means of fluorescing antibodies. *Brit. J. Cancer,* 10, 560–563 (1956). In 11, (922).

WHEATLEY, B. M. An instrument for dosage estimation with fields of any size and shape. *Brit. J. Radiol., N.S.,* 24, 388–391 (1951). In 7, (336).

WHEATLEY, B. M. A method of dose calculation with applications to moving field therapy. *Brit. J. Radiol., N.S.,* 28, 566–573 (1955). In 10, (264).

WHEATLEY, B. M., and others. The two million volt Van der Graaff generator installation designed for rotation therapy at the Royal Cancer Hospital. *Brit. J. Radiol., N.S.,* 26, 57–72 (1953). In 8, (278).

pension contains little or no cell debris—a limitation which does not apply to the agglutination test.

Normal epithelial cells from the urinary tract react in the MCA test strongly and uniformly provided that an excess of antibody is present and that proper mixing is ensured.   An occasional free cell may sometimes be seen but these seldom constitute more than 2 per cent of the total.   Possibly they are stray mesenchymal cells.   When the technique was first tried the cells were trypsinised for one hour and good results were obtained.   Subsequent study has shown that this procedure is unnecessary.

TABLE I.—*Details of 25 Tumours Studied by the MCA Test*

Case	Group	Tissue tested	Histology Deviation from normal Structure	Cellular pleomorphism	Spread	Agglutination	Remarks, etc.
1	A	Bladder primary (biopsy)	+	+	Submucosa only	+ + +	Same tumour re-examined at six weeks interval
2	A	Bladder primary (cystectomy)	+	+	Submucosa only	+ +	Normal mucosa also tested
3	A₁	Bladder primary (biopsy)	+ +	+ +	Muscle + Lymphatics	−	−
4	A	Pleural metastases, fluid aspirate	+ +	+	Widespread metastases	+	Inhibition +. 2-fold. Near diploid tumour.
5	A	Bladder primary (cystectomy)	+	+	Submucosa only	+ +	−
6	A	Inguinal node secondary from bladder	+ +	+ +	? Isolated metastasis 2 yrs after removal of primary	+ + +	+/−
7	A₁	Bladder primary (biopsy)	+ +	+	Muscle + Lymphatics	+	−
8	A₁	Bladder primary (biopsy)	+	+	Submucosa only	+ +	−
9	A	Bladder primary (cystectomy)	+	+	Submucosa only	+ + +	Inhibition 4 fold. Normal mucosa also tested.
10	A₁	Bladder primary (biopsy)	+ + +	+ + +	Muscle + Lymphatics	+ + +	−
11	A₁	Bladder primary (biopsy)	+	+	Submucosa only	+ +	Normal mucosa also tested
12	A	Carcinoma of renal pelvis (nephrectomy)	+	+	Very early infiltration of renal medulla	+ + +	Normal mucosa also tested
13	A	Bladder primary (biopsy)	+ + +	+ + +	Muscle + Interpelvis	+ + +	−
14	A₁	Bladder primary (biopsy and partial cystectomy)	+	+	Submucosa only	+ + + 50%	Inhibition +. 2 fold. Biopsy and cystectomy specimens gave identical results
15	A₁	Bladder primary (biopsy)	+	+	Muscle + Lymphatics	+	−
16	A	Bladder primary (biopsy)	+ + +	+ + +	Muscle (Bladder pelvis in situ)	+ +	−

TABLE I—*cont.*

Case	Group	Tissue tested	Histology Deviation from normal		Spread	Agglutination	Remarks, etc.
			Structure	Cellular pleomorphism			
17	A₂	Bladder primary (cystectomy)	+	+	Muscle + Lymphatics --	+	--
18	A	Pelvic metastasis from bladder (autopsy)	+++	+++	Widespread in pelvis	--	Inhibition *nil*. Normal mucosa also tested. See Table II.
19	A₁	Bladder primary (biopsy)	+++	+++	Lymphatics + Muscle --	++	--
20	A₁	Bladder primary (biopsy)	+	+	Submucosa only	+++	--
21	B	Bladder primary (cystectomy)	+++	+++	Muscle + Lymphatics --	--	Normal mucosa also tested.
22	B	Hepatic metastasis from bladder (autopsy)	+++	+++	Widespread in pelvis and liver	--	Inhibition *nil*.
23	B	Bladder primary (cystectomy)	++	++	Muscle + Lymphatics --	++	--
24	A₁B	Bladder primary (biopsy)	+	+	Submucosa only	A+++ B+++	--
25	A₁B	Bladder primary (biopsy)	++	++	Muscle + Lymphatics +	A+(+) B+++	--
Controls—	A(12) B(6) AB(2)	Epithelium from renal pelvis, ureters and bladder	--	--	--	+++	Inhibition tests: A(4) B(2) c. 4-fold.

Key to Table I

Histology

Structure :	Well-differentiated	+	Cytology : Isomorphic	+
	Poorly-differentiated	++	Slightly pleomorphic	++
	Anaplastic	+++	Markedly pleomorphic	+++

Agglutination

90–100%	+++
50–90%	++
10–50%	+
0–10%	--

Whenever possible normal cells from patients with tumours have been tested along with the tumour cells but for a variety of reasons this could not often be done. In operation specimens, for example, it may be difficult to obtain enough normal cells without stromal contaminants or without a proportion of malignant cells which may be infiltrating the submucosa.

In fact in only six cases could a valid comparison be made. From all of these the normal cells gave a +++ or 90–100 per cent reaction whereas in three of the six (Cases 11, 18 and 22) the tumour cell agglutination was reduced or absent.

Inhibition tests with normal cells again give uniform and consistent results. Thus with a cell concentration of 10,000/c. mm. to which is added an equal volume of homologous serum in dilutions up to 1/128 there is normally an 8-fold inhibition. Non-specific adsorption, however, occurs to account for an inhibiting effect of

WILTSHAW, E., and GALTON, D. A. G. Busulphan (Myleran) in chronic myelogenous leukemia. *Proceedings of the Sixth International Congress of the International Society of Hematology* Boston, 1956. 164–168 (New York, Grune & Stratton, 1958). In **12**, (73)

WINDEYER, B. W., SMITHERS, D. W., and CARLING, E. R. Cancer of the breast. *British Practice in Radiotherapy*. Ed. Carling, Windeyer and Smithers. 129–148 (London, Butterworth, 1955). In **10**, (151).

WORSTER-DROUGHT, C. Blood-vessel tumours of the brain. *Post Grad. med. J*, 27, 160–165 (1951). In **7**, (162).

WORTHLEY, B. W., and WHEATLEY, B. M. A generalised method of rapid dosage estimation with particular reference to 200 KV therapy. *Brit. J. Radiol., N.S.*, 25, 491–501 (1952) In 8, (152).

WYBURN-MASON, R. Malignant change arising in tissues affected by herpes. *Brit ed J*, ii, 1106–1109 (1955). In **10**, (60).

WYBURN-MASON, R. Malignant change following herpes simplex. *Brit. med. J.*, ii, 615–616 (1957) In **12**, (198).

WYBURN-MASON, R. Visceral lesions in herpes zoster. *Brit. med. J.*, i, 678–681 (1957) In **12**, (194)

Agglutination of individual cells does not appear to be an all-or-none phenomenon. Weak positive reactions usually include some completely agglutinated cells but also many cells with a reduced complement of adherent erythrocytes and a correspondingly large area of free surface.

In only one of the negative results has there been complete absence of agglutination. In the other four occasional agglutinated cells, about 1-2 per cent, were noted. It would be easy to ascribe these to artefact were it not that they do not occur in any of the control tubes. Inclusion of a few normal cells is unlikely as great care was taken to sample the most central part of the primary tumour surface. Furthermore they were present in suspensions from metastases.

DISCUSSION

An analysis of the results as presented in Table I reveals an approximate inverse relationship between the degree of agglutination and the malignancy of the tumour as judged both by microscopy and by the extent of infiltration or metastasis (Table III)

TABLE III —*Relation of MCA Test to Structure and Spread of Bladder Carcinomata*

		Agglutination		
		+++	++ and +	0
Spread	Submucosa only .	5	4	0
	Lymphatics and/or bladder musculature	3½	4½	3
	Metastases	1	2	2
Histology	Well-differentiated	6	5	1
	Poorly-differentiated	1½	3½	1
	Anaplastic .	2	2	3

If anything the correla·   ᵗʰ ᵗᵘᵐᵒᵘʳ-spread is the closer of the two. Thus no tumour limited to ·   ·   ·⁻ᵗⁱᵒⁿ reaction and only one metastasis ga·e   :   · · ¹·   · · 'eposit in an inguinal lymph node occurring some two years after removal   · · primary vesical carcinoma, there being no other evidence of recurrence. On the other hand one well-differentiated tumour was totally unagglutinable while two anaplastic pleomorphic growths retained normal reactions.

It would seem from the inhibition tests that the negative results were due to inability of the malignant cells to adsorb antibody rather than to failure at a later stage of the agglutination procedure, e.g. inhibition of adsorbed antibody or detachment of loosely bound antibody during washing. It remains to explain this phenomenon.

It is possible but unlikely that there is actual loss of the genes responsible for A and B antigens due to some chromosomal rearrangement in the tumour cells. In favour of this explanation is the behaviour of one of the AB tumours in which a definite distinction was apparent between the A and B reactions. On the other hand two of the pleomorphic tumours, which might be expected to give rise to chromosome-deficient cells, gave normal reactions. Possibly they arose in subjects homozygous for the A gene. A stronger argument against the hypothesis of gene-loss lies in the gradation of reduced agglutinability not so much in cell populations as in individual cells. Genic loss should produce an all-or-none effect which, as has been noted, does not occur.

slightly under 2-fold so that the net specific adsorption is in the region of 4-fold (Table II). This has been demonstrated in six cases, four of group A and two of group B.

TABLE II.—*Correlation of MCA Test and Antibody Adsorption test.*
Negative Result in Latter Test Indicates Adsorption.

Autopsy (4 hours post mortem) samples of normal ureteric epithelium and secondary deposit of anaplastic bladder carcinoma in pelvis (Case 18)
Blood Group A.
Ureteric suspension : 9600 cells/c. mm.
Tumour suspension : c. 9000 cells/c. mm.   Accurate counting impossible.

	Agglutination test					
	Ureter			Tumour		
	A cells.	B cells.			A cells.	B cells.
Anti A	+ + + (100%)	—	.	.	— (2%)	—
Anti B	—	—			—	—

	Antibody adsorption test					
	Anti A			Anti B		
Serum dilution	Control	Ureter	Tumour	Control	Ureter	Tumour
Neat	+ + +	+ +	+ + +	+ + +	+ + +	+ + +
1/2	+ + +	w	+ + +	+ + +	+ + +	+ + +
1/4	+ + +	—	— +	+ + +	+ +	+ +
1/8	+ +	—	+	+ + +	+ +	+ +
1/16	+ +	.	—	+ + +	w	+
1/32	—	.	.	w	.	—
1/64	—	.	.	—	.	.

In five tumour suspensions subjected to inhibition tests it appeared, not altogether unexpectedly, that loss of inhibiting activity ran parallel to loss of agglutinability.

Histologically an attempt was made to divide the tumours into three grades of malignancy on two separate criteria, one of structural deviation from the normal and one of cellular irregularity or pleomorphism. With two exceptions these were found to give the same grading. In one case (Case 4), where cells were obtained by aspiration of pleural fluid, a chromosome analysis was performed using the Feulgen squash technique of Ford and Hamerton (1956). Thirty cells counted to an accuracy of ± 3 contained 40-50 chromosomes and some of these were more definitely near the middle of this range. In other words there was no polyploidy, as was already suggested by the uniformity of the non-mitotic cell nuclei.

In using the agglutination technique the degree of agglutination was at first indicated by a simple system based on numbers of + signs. Subsequently counts of agglutinated and unagglutinated cells have been performed where possible for the sake of greater precision. Negative and strongly positive (+ + + or + + + +) per cent results can be taken as reasonably accurate. Intermediate results have shown some variability (i.e. 20 per cent inhibition grading there was a wide range in this criterion, e.g. Case 18 where both ureter and subsequent cystoscopic specimens showed almost exactly 50 per cent agglutination.

RADIOTHERAPY OF NON-MALIGNANT
DISEASES OF THE EYE*

BY

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RADIOTHERAPY has been used in the treatment of non-malignant disease of
the eye for over 50 years; but progress in the development of this form of
treatment has been hampered by its alleged dangers to the eye. It is true
that in the early days ocular damage was frequently encountered, but the
traditional view that the eye is readily damaged by radiation, was based upon
early unsound experimental work, and the memory of the clinical disasters
encountered by the pioneers. To-day this view can no longer be maintained,
for with reasonable technical care and proficiency the radiation treatment of
non-malignant disease of the eye should entail no risk of ocular damage
either as a complication or as a sequela.

PURPOSE OF RADIOTHERAPY

In assessing the value of and indications for radiotherapy in non-malignant
diseases of the eye, the purpose or object of this form of treatment must
be clearly appreciated; otherwise the treatment may be misapplied, so that
the patient is not benefited and the method itself becomes discredited.

(A) NON-NEOPLASTIC DISEASE.—In such cases the main aims of radiation
treatment are:

    (1) To relieve symptoms, particularly pain.

    (2) To promote healing by assisting:
        (i) the resolution of inflammatory processes,
        (ii) the absorption of a haematoma or haemorrhage;
        (iii) the epithelization of an ulcerated surface;
        (iv) the organization and removal of granulation tissue.

    (3) To affect blood vessels:
        (i) to reduce vascular engorgement and congestion accompanying inflammation;
        (ii) to obliterate or reduce in size newly-formed vessels invading the cornea.

    (4) To reduce intra-ocular tension.

Alternatively and more probably there occurs some general change in the surface of the cell which masks the A and B antigens. In support of this view one may quote a variety of related phenomena. Irregularity of the surface as viewed by electron microscopy (Coman and Anderson, 1955) a deficiency in the capacity to bind calcium (Dunham, Nichols and Brunschwig, 1946; de Long, Coman, and Zeidman, 1950), decreased adherence (McCutcheon, Coman and, Moore, 1948), absence of contact inhibition (Abercrombie and Heaysman, 1953, 1954) and accelerated electrophoretic mobility (Ambrose, James and Lowick, 1956) have all been demonstrated. However the fundamental change underlying these has not yet been elucidated, and it remains to be shown whether any or all of them run parallel to the apparent changes in antigen-content.

The observations here presented may also be relevant to the problem of antibody-resistance in tumours. It seems probable that antigens are not entirely lost by tumour cells since they remain demonstrable to a greater or lesser degree in a proportion. Many cells on the other hand might be able to resist the effects of antibody owing to reduced adsorption at their surface.

On a more practical plane it may be that the method described will give useful information relating to the malignancy of the tumour and the probability of infiltration. A drawback here is the immunity of group O cells to antibodies that are easily available. Eel serum anti-H, for example, is ineffective in the test.

SUMMARY

The epithelium of the human urinary tract contains A and B antigens which are easily demonstrable by the MCA test.

Tumours arising from this epithelium may fail to exhibit these antigens. The implications of this are discussed

REFERENCES

ABERCROMBIE, M AND HEAYSMAN, J. E M—(1953) *Exp Cell Res.*, **5**, 111 —(1954) *Ibid.*, **5**, 293
AMBROSE, E J, JAMES, A. M AND LOWICK, J. H. B.—(1956) *Nature*, **177**, 576.
COMAN, D. R. AND ANDERSON, T. F —(1955) *Cancer Res.*, **15**, 541.
COOMBS, R. R A., BEDFORD, D AND ROUILLARD, L M.—(1956) *Lancet*, **i**, 461
DUNHAM, L. T., NICHOLS, S. AND BRUNSCHWIG, A —(1946) *Cancer Res.*, **6**, 233.
FORD, C. E. AND HAMERTON, J. L —(1956) *Stain Tech*, **31**, 247.
GREEN, H N.—(1954) *Brit med J.*, **ii**, 1374.
DE LONG, R P, COMAN, D. R. AND ZEIDMAN, I —(1950) *Cancer*, **3**, 718.
MCCUTCHEON, M., COMAN, D R AND MOORE, F. B.—(1948) *Ibid.*, **1**, 460.
WEILER, E —(1956) T. *Naturforsch.*, **11B**, 31—(1956) *Brit J Cancer*, **10**, 553, 560.

TABLE I
NON-NEOPLASTIC LESIONS

Inflammatory Processes	Pyogenic	Blepharitis
	Granulomatous	Tuberculosis Sarcoidosis
	Allergic	Vernal catarrh Phlyctenular kerato-conjunctivitis
	Viral	Superficial punctate keratitis Post-herpetic pain Dendritic ulcer Disciform keratitis Trachoma
	Unknown aetiology	Rosacea keratitis Marginal ulcerative keratitis
Miscellaneous	Corneal lesions	Ulcers (including Mooren's) Opacities following infection or trauma Dystrophies and degenerations (including pterygium) Traumatic lesions and recurrent erosions
	Epithelial downgrowths	
	Vascular abnormalities	Corneal vascularization (particularly after grafting) Eales's disease Retinal haemorrhage Venous thrombosis
	Raised intra-ocular tension	Secondary glaucoma

Radiotherapy should therefore be considered in the following circumstances:

(1) Where there is no response to recognized therapeutic methods within a reasonable time (1 to 2 months).

(2) Where no accepted method of treatment exists and there is reason to believe that radiotherapy may be of help.

(3) For chronic progressive lesions accompanied by symptoms which radiotherapy may relieve without necessarily arresting the course of the disease.

(4) For chronic relapsing lesions wherein periods of apparent improvement or healing are followed by repeated recurrence.

Of the conditions listed in Table I, the following are of most importance and interest to the radiotherapist:

(1) Mooren's ulcer.
(2) Rosacea keratitis.
(3) Corneal vascularization.
(4) Virus infections.
(5) Vernal catarrh
(6) Pterygium.

(5) To restore function. *E.g.* an attempt may be made to improve vision where the cornea is the seat of an opacity, dystrophy, or degenerative process.

(B) NON-MALIGNANT TUMOURS.—In such cases radiation can be employed for the following purposes:

(1) To destroy a tumour for cosmetic reasons.

(2) To destroy or hasten the natural resolution of a tumour adjacent to and endangering the eye.

(3) To eradicate a precancerous condition.

The precise mode of action whereby radiation can favourably influence an inflammatory process is not known, but there are three possible mechanisms which may separately or together play some part in producing a favourable response (Pohle, 1950):

(1) A possible stimulation of antibodies

(2) An effect on the cellular elements of the exudate, particularly the leucocytes.

(3) An effect upon the blood vasculature, especially the capillaries.

In the case of the benign tumours, the mode of action of radiation is by selective destruction of the tumour tissue, and it presumably differs in no fundamental way from the mode of action of radiation on other neoplastic processes.
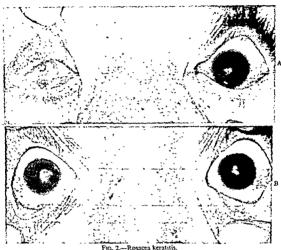
INDICATIONS AND SELECTION OF CASES FOR RADIOTHERAPY

(A) INFLAMMATORY AND OTHER NON-NEOPLASTIC CONDITIONS

Whilst the exact mode of action of radiation in these conditions is uncertain and further speculation on this topic would be profitless, the fact remains that a sound knowledge of the clinical effects of radiotherapy and the purposes to which these can be put, provide a reasonable basis for selecting cases for this form of treatment. Table I (opposite) lists the non-neoplastic lesions, in the treatment of which experience shows radiotherapy to be of value.

It is obvious that only a minority of patients suffering from these diseases is likely to require radiotherapy, and an indiscriminate resort to this form of treatment is to be deprecated. Radiation is, after all, a noxious agent, its precise mode of action is still a matter for conjecture, and its effects are neither prompt nor certain. In these circumstances, any other methods of treatment known to give satisfactory results should always be given priority.

Whilst a hasty resort to radiotherapy is not to be recommended, undue tardiness is equally to be avoided. A patient failing to respond to recognized therapeutic measures within a reasonable time should not be deprived of possible help from radiotherapy, nor should he be left until the disease progresses to the stage when radiotherapy can only give symptomatic relief.

FIG. 2.—Rosacea keratitis.
A. Before radiation treatment.   B. After radiation treatment.

FIG 3.—Lamellar graft.
A. Before radiation treatment.   B. After radiation treatment.
(By courtesy of the Editors of the *British Journal of Radiology*.)
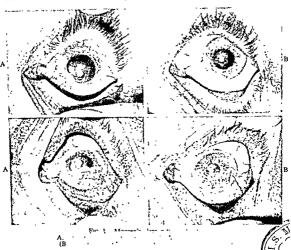
(5) A note of warning must be sounded in relation to the radiation treatment of vernal catarrh. It has been known for a very long time that radiotherapy can help sufferers from this condition, but in recent years there has been an enthusiastic revival of the beta-ray treatment of this disease, particularly in the United States. Although the sufferers are usually

(1) In cases of Mooren's ulcer, radiotherapy is the only method of treatment known to be of value (Fig. 1).

A.
(B

(2) There are very few remedies that can compare with radiotherapy in the treatment of rosacea keratitis, where radiation virtually proves itself the "morphia of the eye" (Fig. 2, opposite).

These two lesions are the sole exceptions to the general rule that radiotherapy should be used only as a last resort in the treatment of non-neoplastic processes. The early use of radiotherapy in Mooren's ulceration gives the patient his only chance of cure; in the treatment of rosacea keratitis, it gives the patient his best chance of avoiding loss of vision through the scarring that follows repeated attacks.

(3) The judicious use of radiation for corneal vascularization associated with keratoplasty can go far to help obtain a satisfactory functional result as in Fig. 3 (opposite) and Figs 4 and 5 (overleaf).

(4) In virus infections (Fig. 6, overleaf), and superficial punctate keratitis (Fig. 7, overleaf), radiotherapy is only occasionally curative, but it can nevertheless effect a certain degree of symptomatic relief not to be obtained by other remedies (Lederman, 1956b).

FIG. 6.—Dendritic ulcer   A Before treatment.   B After beta-radiation.
Second attack 5 years later.   C. Before treatment.   D. After beta-radiation
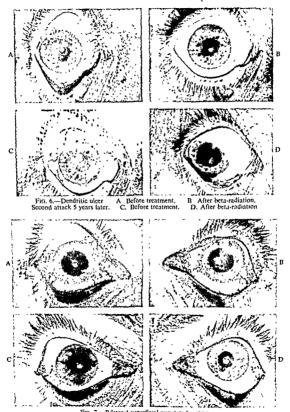
FIG. 7.—Bilateral superficial punctate keratitis.
A and B. Before treatment   C and D After small doses of x-ray therapy.
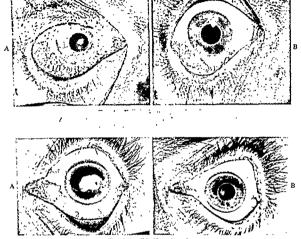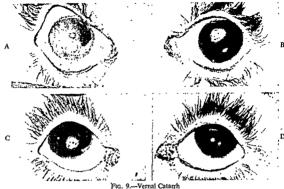
FIG. 5 A.—Disciform keratitis
B Full-thickness graft in association with pre- and post-operative irradiation.
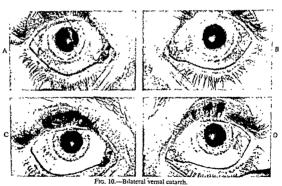
children and the disease is seasonal and ultimately self-limiting, high doses of beta-radiation have been recommended as a method of eradicating the characteristic "cobble-stone" papules that appear on the tarsal conjunctiva. To any radiotherapist conversant with the effects of beta-radiation, the consequences of high doses applied to the mucosal surfaces of the lids could easily be predicted—namely, replacement fibrosis with atrophy and scarring of the palpebral conjunctival mucous membrane. Merriam (1956) has pointed out the risks of irradiating vernal catarrh in a recent paper on the effects on the eye of beta-radiation. He gives the following graphic account of the state of the unfortunate sufferers from this disease who have been misguidedly submitted to over-enthusiastic beta-irradiation:

When the roughened keratinized surface (of the lid) rubs over the cornea, it produces a punctate keratitis with severe photophobia, lacrimation, and blepharospasm. This persists throughout the patient's lifetime, is resistant to treatment, and in our experience has been found to be much more debilitating than the disease originally treated. This possible effect should be given serious consideration before irradiation is advocated for a condition in which 90 per cent. of the cases subside spontaneously with local medication.

FIG. 9.—Vernal Catarrh

A and B. State of eyes before small doses of x-ray therapy—corneal damage is present.
C and D. State of eyes after *treatment*—the eyes are white and quiet and there has been relief
of symptoms.

FIG. 10.—Bilateral vernal catarrh.

A and B Bilateral bulbar vernal catarrh before treatment.
C and D After beta-radiation—symptomatically improved.

The present writer has not practised the beta-ray treatment of vernal catarrh using high doses; after tentative trials, when it became obvious that the papules could not be eradicated with small safe doses, beta-radiation was virtually abandoned and small doses of x-radiation were used instead. The aim of treatment has been either prevention by irradiation one month before the expected onset of the seasonal attack, or reduction of the acuteness of the florid attack to relieve symptoms until the seasonal respite occurs. Beta-radiation is indicated only in the rare cases in which the bulbar conjunctiva is affected (Figs 8, 9, and 10).
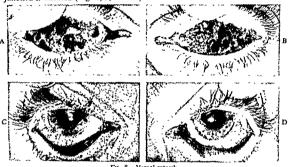
FIG 8.—Vernal catarrh.
A and B Typical "cobble-stone" appearance of lids
C and D. Associated corneal ulceration.
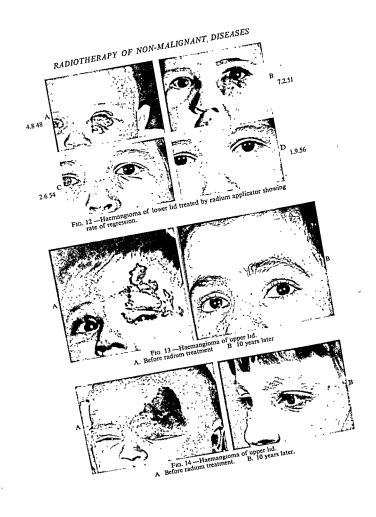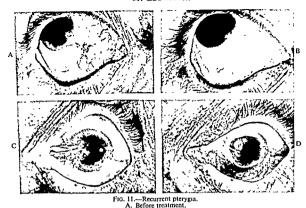
(6) Most of the cases of pterygium seen by the writer have been in patients coming from tropical and subtropical countries. The customary treatment is surgical removal, but the recurrence rate is by no means negligible. Radiotherapy should be reserved for the recurrent case, when excision and immediate post-operative radiation is the treatment of choice (Fig. 11, overleaf).

(B) BENIGN TUMOURS

The non-malignant tumours of the outer eye for which radiotherapy may be indicated are haemangiomata, papillomata, hyperkeratoses, and keloids. The need for and value of radiotherapy varies for each lesion.

(1) Haemangiomata.—It is probably true that most haemangiomata occurring in infancy disappear spontaneously without treatment. In spite of this, however, there are certain medical indications for active treatment:

(i) Presence of haemorrhage or infection.

FIG. 12.—Haemangioma of lower lid treated by radium applicator showing rate of regression.

FIG 13.—Haemangioma of upper lid
A. Before radium treatment   B. 10 years later

FIG. 14.—Haemangioma of upper lid.
A Before radium treatment.   B. 10 years later.

FIG. 11.—Recurrent pterygia.
A. Before treatment.
B After treatment
Treatment was by surgery and immediate post-operative beta-radiation.

(*ii*) Situation of the haemangioma in a region exposed to frequent trauma,
i.e. the scalp or the napkin area, or where its presence predisposes to
accept infection of a neighbouring organ; *e.g.* infection may take place in the
conjunctival sac where the lids are closed by an angioma.

In addition to these medical indications there is one further important
indication; namely, the child is treated for the mother's sake. Many of the
sufferers are first-born girl babies, and it is unreasonable to expect the mother
to accept verbal reassurance that all will be well, particularly during the
period when the angioma apparently increases in size with the growth of
the infant. Radiotherapy is the treatment of choice for lid angiomata.
There is no indication in these cases for excisional surgery which can only
result in unnecessary mutilation and should be avoided at all costs. Radio-
therapy offers a simple and safe remedy, the judicious use of which can
arrest the growth of the angioma and restore confidence and peace of mind
to the parents (Figs 12, 13, and 14, opposite).

(2) Papillomata.—The treatment of choice for either the true papilloma,
or the infective wart is surgical removal. Such warts may be single or
multiple and may affect the lids or the conjunctiva. It is rarely possible to
cause true papillomata to disappear by using small safe doses of radiation,
and an adequately effective dose of radiation must inevitably be accompanied

Fig 16.—Multiple papillomata of the lids and bulbar conjunctiva treated by a combination of low-voltage x-rays to lid and beta-radiation to the bulbar conjunctiva.
A. Before treatment.   B. After treatment.

Fig 17.—Papilloma of the conjunctiva.
A. Before radiation treatment.   B. After radiation treatment.

(4) Keloids.—These may form in a scar on the lid, or may occur after injury or post-operatively. If the keloid is of the raised, red, fleshy type, radiotherapy should be undertaken, but if the lesion is of the hard, white, linear variety, excision associated with pre- or post-operative irradiation is indicated.

FACTORS INFLUENCING CHOICE OF TECHNIQUE

(A) NON-NEOPLASTIC LESIONS

The one factor which must dominate treatment is safety; in no circumstances must risks of damage be taken. Treatment can be made safe by attention to technical detail and utmost care in dosage.

The superficial situation and accessibility of the outer eye considerably simplifies apparatus requirements. With the exception of irradiation of the Gasserian ganglion for post-herpetic pain, which requires high-voltage therapy, all other non-neoplastic lesions can be treated by low-voltage therapy or beta-radiation, used either separately or in combination.

The particular method to be used chiefly depends on the acuteness and the extent of the lesion.

by a local tissue reaction. Such reactions occurring in regions away from the eye are reasonably free from risk, but in the eyelid or on the conjunctiva there are three special complications, the risk of which is real enough to contraindicate radiotherapy:

    (i) Keratinization of the conjunctival epithelium of the treatment area. This occurs rarely but when it does it can be a serious matter, since, apart from producing an irritable eye, it may also damage the cornea (Fig. 15).

FIG. 15.—Papilloma of upper lid.
A. Before treatment. B. After treatment. The lesion has gone but there is keratinization of the lid conjunctiva in the treated area.

    (ii) In the writer's experience a single-dose technique (2,000 r by L.V.T. 60 kV.) tends to produce a higher necrosis rate than fractionated techniques. The reason for using these single-dose techniques is their simplicity and economy, but radio-necrosis is a high price for a patient to pay for the treatment of a non-malignant lesion for which a safe surgical alternative is available.

    (iii) The x-ray treatment of an epibulbar wart, particularly if situated near the limbus, may be followed by a radiation cataract. This risk can be minimized by the use of beta-radiation.

In spite of the desire to use surgery whenever possible, there are certain indications for radiotherapy in the treatment of papillomata:

    (i) For a solitary sessile papilloma affecting more than one-third of the lid margin, when surgery would involve plastic repair of the lid (Fig. 15).
    (ii) Multiple papillomata of the lids and conjunctiva (Figs 16 and 17, opposite).
    (iii) Repeated recurrences after surgery.

    (3) Keratoses.—The senile type of keratosis is usually a precancerous lesion and when affecting the lid margin is best treated by radiotherapy, because a wider area of tissue can be irradiated than can be excised. The need to irradiate or excise a wide area of tissue surrounding the lesion is due to the presumed "instability" of the tissues from which the keratosis arises.

X-ray treatment is given once or twice a week for 4 weeks, and the treatment can be stopped if the eye is comfortable and quiet at the end of this period. If the eye is white and corneal ulceration persists, beta-radiation can be used; if no improvement is observed, radiation treatment should be abandoned completely.

(2) *Beta-Radiation* —This forms the mainstay of the treatment of corneal lesions. The apparatus comprises a plastic shell containing radio-active strontium foil (Lederman, 1956a). The shell is inserted into the conjunctival sac so that the radio-active surface comes into contact with the cornea and the surface of the globe. A range of such shells is available (Figs 18 and 19).

FIG. 18.—Radio-active strontium beta-ray applicator in place in conjunctival sac.
(By courtesy of the Editors of the *British Journal of Radiology*.)

FIG. 19.—Radio-active strontium beta-ray applicators showing range of applicators available.
(By courtesy of the Editors of the *British Journal of Radiology*)

The dosage required depends on the lesion being treated.

For Mooren's ulcer single doses of 500 to 1,000 r are given at weekly intervals for 4 weeks.

For corneal ulceration of dendritic or rosacea origin a dose of 500 r is given weekly.

For corneal vascularization after grafting treatment may be begun 10 to 14 days after operation, and 500 to 1,000 r are given weekly for 4 weeks. The dose and the type of applicator used depend on the state of the eye when seen, and the extent,

(1) Low-voltage therapy is *always* used for an acute or extensive lesion. The radiation is applied directly to the closed eye so that the whole of the outer eye and the eye-ball are irradiated.

(2) Beta-radiation is reserved for chronic lesions, chiefly affecting the cornea. Beta-radiation is not used in the presence of:

  (a) severe lid spasm,
  (b) a small pupil, marked ciliary congestion, or other evidence of uveal irritation or inflammation.

  In the presence of lid spasm, any difficulty in inserting the beta-ray shell into the conjunctival sac may result in injury to the cornea, and the strontium beta-ray dose delivered to any co-existing inflammatory process deep to the cornea might prove excessive and produce an exacerbation of symptoms.

(3) Quite frequently, x- and beta-radiation may be used in combination. Thus, for an acute kerato-conjunctivitis or a deep keratitis associated with an iridocyclitis, treatment can be begun with low-voltage therapy and when the eye is reasonably comfortable and white, beta-rays can be applied to any persistent localized corneal lesion.

The effect desired from the treatment governs the selection of the particular radiation and dosage used. The purpose of the x-ray treatment is to assist the resolution of an acute inflammatory process affecting a part or the whole of the eye. The radiation is, therefore, applied in very small doses to the affected organ in its entirety on the assumption that the favourable effect of the radiation mediates through the blood supply, all of which should therefore be exposed to the radiation. In the case of beta-radiation, the "anti-inflammatory effect" of radiation is not usually sought, but the purpose is to heal a corneal ulcer or obliterate unwanted blood vessels invading the cornea; to achieve this, the strictly localized effects of high doses of beta-radiation are ideal.

There are two standard techniques for treating non-neoplastic lesions of the eye.

(1) *X-Radiation: Bulbar or "Whole Eye" Technique.*—This is so-called because no attempt is made to protect or shield any part of the globe or lids, treatment being given through a 3-cm. diameter circular field placed directly over the closed lids. The apparatus used has the following factors: 45 kV., 2 mA ; filter 1 mm. Al; focal-skin distance 4 cm.

The x-ray dosage employed in the treatment of an infection of the outer eye is always minimal, namely 10 r, and it would appear that, if radiotherapy is going to help, this minute dose is perfectly adequate. In my experience, there is no advantage in giving higher doses as, for example, of 100 r: the results are the same, the smaller dose is absolutely safe, does not provoke exacerbations, requires no manipulation of the lids, and can be continued for more than a month with safety. In the event of a relapse, a course of low-dosage treatment can be repeated two or three times a year.

Gamma-radiation is to be preferred, but, because of the greater ease in protecting the eye, contact therapy (60 kV.) is usually employed for lid angiomata, and the child is anaesthetized for the treatment in most cases. A single dose of 250–400 r is given, depending on the age of the patient and the thickness of the lesion, the lower dose never being exceeded in a newborn infant. The treatment is not repeated as long as regression continues, but if the improvement is not maintained a further treatment is given after 6 months, and very rarely a third treatment after 18 months. The child should be treated as soon after birth as possible and the parents warned that complete regression may not be obtained for from 3 to 5 years or more (Fig. 12).

(2) *Papillomata.—X*-ray therapy is used for a solitary papilloma, a single dose of 2,000 r at 60 kV. being given. Infective or multiple papillomata in children are treated by *x*-ray therapy to the lid lesions, a single dose of 600 r being given, and beta-ray therapy to any bulbar conjunctival lesion, a single dose of 1,000 r being given. These doses can be repeated at the end of 3 months if necessary.

(3) *Keratoses.*—The technique is the same as for solitary papilloma.

(4) *Keloids.*—A single dose of 600 r, using *x*-rays at 60 kV. or 100 kV., is given to the scar and treatment is repeated in 3 months if necessary.

COMPLICATIONS OF RADIOTHERAPY

Complications should not be encountered in the radiation treatment of inflammatory processes.

(1) Exacerbation of symptoms during radiotherapy is nearly always a sign of improper dosage; in using *x*-ray therapy, the minute doses given never provoke exacerbations such as may occur with higher doses.

(2) Infection and traumatic damage to the eye may occur when using beta-rays, but with proper care these complications are entirely avoidable. Merriam (1956) has reported on the occurrence of radiation cataract after strontium beta-radiation. He notes that damage to the lens can occur only with very high surface doses. There are no indications for very high beta-ray dosage, *i e* over 4,000 to 5,000 r, in the treatment of non-neoplastic disease, and to date we have no record of a radiation cataract produced by beta-rays.

(3) Radionecrosis has occurred only after the single-dose method of treating lid tumours. A fractionated dose method is clearly preferable if radiotherapy is to be used safely rather than economically.

SUMMARY

In assessing the value of radiotherapy in the treatment of non-malignant disease, it is important to remember the following facts:

(1) Radiotherapy is not a specific remedy.

(2) Many of the lesions treated are normally capable of spontaneous healing, but may relapse in a certain unknown proportion of cases.

age, and depth of the vessels to be treated; the treatment is the same whether the graft be lamellar or full-thickness. For pre-operative corneal irradiation, doses of the order of 500 to 800 r weekly are given for 2 to 4 weeks, and operation is advised 10 to 14 days after the last treatment. Pre-operative radiation can be followed by post-operative treatment in the more heavily vascularized cases.

For vernal catarrh, the patient should ideally be treated one month before the expected onset of the attack. A lead shield is placed in the conjunctival sac to protect the eye and the size of field used will depend on whether one or both lids are to be treated, in each case the fornices must be irradiated and the field extended to the bony orbital margin. A dose of 100 r is given once weekly for 4 weeks. If the patient is seen during an attack, the same treatment should be given. It is advisable when treating any bilateral eye condition to begin by treating the worse eye first and to treat both eyes only if no exacerbation of symptoms has occurred. For bulbar lesions, beta-radiation should be used in order to avoid lens damage, a dose of 500 r being given once a week for 4 weeks.

Recurrent pterygium can be treated by beta-radiation using the strontium shell, four doses of 500 to 600 r being given at weekly intervals It can also be treated post-operatively by low-voltage x-rays (60 kV.) given through a 2×1-cm, field, so placed that its edge is at the apex of the pterygium and tangential to the cornea. The eye is anaesthetized and a lid-retractor is used. A single dose of 600 r is given, and this can be repeated after 6 weeks if necessary

The doses used in beta-ray therapy bear little relation to the doses employed in low-voltage x-ray therapy, for the following reasons:

(i) The marked difference in penetration of the two types of radiation   Under our treatment conditions, the dose of x-radiation incident on the anterior surface of the lids falls to half its intensity at a depth of 1·1 cm. In contrast, the dose of strontium radiation incident on the surface of the cornea falls to half its intensity at a depth of 1·3 mm.

(ii) The volumes of tissue irradiated by each method vary greatly. With low-voltage therapy the lids and the eye itself are irradiated, whereas with beta-radiation only tissues in close contact with the source are irradiated. There is an inverse relationship between the volume of tissue irradiated and the size of the dose, *i.e.* the larger the volume the smaller the dose that can be safely given.

Any attempt to imitate the high single doses used in beta-ray therapy with low-voltage x-ray therapy would almost certainly be harmful to the inflamed eye. Conversely, to imitate the small doses used in x-ray therapy with a beta-ray source would be pointless and ineffective.

(B) Benign Tumours

(1) Haemangiomata.—It is our general policy:

(i) not to give large doses of irradiation to these tumours,
(ii) to avoid radon seed or other implantation methods;
(iii) to repeat treatment only once or twice with the longest possible intervals between treatments.

TABLE VI
MOOREN'S ULCER

Treated		Healed	Improved	Failed or too Recent
Patients	34	11	10	13
Eyes	45	15	14	16

TABLE VII
PTERYGIA

Seen		Treated			Not Treated
		Successful	Improved	Failed	
Patients	12				
Eyes	19	3	7	3	6

TABLE VIII
CORNEAL GRAFTS

Radiation Treatment	Number Seen		Satisfactory		Failed		Indeterminate	
	Patients	Eyes	Patients	Eyes	Patients	Eyes	Patients	Eyes
Post-keratoplasty Lamellar Full-thickness	67	70 26 44	21	22	22	23	24	25
Pre-keratoplasty	18	19	2	2	10	10	6	7
Pre- and Post-keratoplasty	5	7	2	2	2	3	1	2
Total	90	96	25	26	34	36	31	34

REFERENCES

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(3) Radiation is often used only when all else has failed and patients often come with a chronic long-standing lesion or in an acute " resistant " stage.

(4) Because of the benign or self-limiting nature of many of the conditions treated, routine follow-up is not undertaken. A detailed statistical analysis of results, such as is available in the treatment of malignant diseases, is scarcely indicated.

Tables II to VIII show the results of treatment obtained at the Royal Marsden Hospital in a series of cases of various kinds.

TABLE II
HAEMANGIOMATA AND PAPILLOMATA

Condition		Seen	Treated		Not Treated
			Improved	Failed	
Haemangiomata...	Lids	50	45	2	3
	Canthi	7	4	—	3
Papillomata ..	Lids	41	27	3	11

TABLE III
VERNAL CATARRH

Seen	Treated		Not Treated
	Improved	Failed	
21	15	2	4

TABLE IV
VIRUS INFECTIONS OF THE OUTER EYE

Infection	Seen	Treated			Not Treated
		Relieved	Improved	Failed	
Dendritic ulcer	22	3	10	7	2
Disciform keratitis	17	3	7	6	1
Post-herpetic pain	11	2	3	6	0
Superficial punctate keratitis ...	73	12	44	12	5

By courtesy of the Editors of the *Transactions of the Ophthalmological Society of the United Kingdom*

TABLE V
ROSACEA KERATITIS

Treated	Healed	Improved	Failed or too Recent
101	25	68	8

treated at the Royal Marsden Hospital, there were nine orbital exenterations and only one patient is alive a year later. Of eight other patients who suffered enucleation of the eye (in no case was removal necessary because of radiation damage) there are two living for more than three years; in both cases the patients came into the hands of plastic surgeons; one eye was removed for infection, and the other in spite of advice to the contrary.

(*c*) *The purpose of treatment.* Whenever curative treatment is undertaken every attempt must be made to limit the permanent damage inflicted on the eye. The discomforts associated with severe reactions must be accepted in the knowledge that with proper care and attention they should be temporary. When, however, palliation alone is the objective, as with metastatic orbital tumours from the breast and bronchus, the whole globe should always be protected from direct irradiation and reactions minimised as much as possible by control of dosage. The purpose of treatment in these cases is to relieve pain and proptosis so as to enable the patient to die without having to undergo an operation on the eye or to endure the miseries of uncontrolled proptosis.

(2) *Of the local factors* affecting technique the chief are the nature and extent of the tumour and its situation within the orbit.

(*a*) *Benign tumours and granulomata.* When the tumour is restricted to one part of the orbit, radiation can be localised to the affected region. Treatment can be given through a single anterior field applied directly to the eye with some form of protection to be described later. The eye must be completely protected and no risk of damage taken.

(*b*) *Primary malignant tumours.* When dealing with these tumours, the technique of treatment must be such that the whole orbit is irradiated. There are only two exceptions to this rule:

(i) The pre- or post-operative treatment of a lacrimal gland tumour, when treatment can be localised to the region of the lacrimal fossa, care being taken to irradiate a wide zone of the surrounding bone because of the known tendency of these tumours to produce early and widespread bone invasion.

(ii) In the treatment of glioma of the optic nerve or other rare tumours affecting the posterior part of the orbital cavity, the radiation can be largely concentrated in the retro-ocular segment of the orbit, the posterior part of the globe related to the nerve head alone being included in the field of radiation.

In all other cases where the whole orbit has to be irradiated the inclusion of the eye to a greater or lesser extent is unavoidable unless one is prepared to

run the risk of leaving some part of the tumour inadequately irradiated.

In treating a primary orbital tumour two fields (an anterior and a lateral) are usually necessary, although usually a single direct field can be employed for some of the very sensitive sarcomas of lymphoid tissue. The field sizes used are 4×4 cm² minimum, the exact size depending on the extent of the tumour, and for the purposes of beam direction the fields are mounted on wax seats on a plaster cast if 220 kV X rays are used (Fig. 1), or if 2 MeV

FIG. 1.

Treatment of an orbital tumour at 220 kV using a cast. Note protection of the sound eye.

radiation is employed the fields are outlined on a Perspex cast on which are also mounted the points at which the central axes of the beams emerge (Fig. 2). The use of a light device instead of a close-ended applicator is of particular value, since it is atraumatic and is the only technical method of administering radiation which permits the use of the indirect method of eye protection to be described later.

(*c*) *Bilateral orbital tumours.* The rare bilateral tumours of the orbit are usually radiosensitive sarcomas of lymphoid tissue, and the technique of treatment should be the same for both orbits. It is wise to begin treatment on the most seriously affected side and allow a few days interval before beginning treatment to the second tumour.

(*d*) *Secondary orbital tumours.* The important secondary orbital tumours are those due to extension of neoplasm from the adjacent paranasal sinuses or postnasal space. The two cardinal principles to be observed in treating secondary orbital invasion from these regions are:

(i) The treatment technique must be predominantly directed towards control of the primary site and the orbital extension coincidentally and adequately included within the treatment zone.

(ii) The treatment can be limited to the part of the orbit affected, provided the direction of invasion is

TECHNIQUE OF RADIATION TREATMENT OF ORBITAL TUMOURS*

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PRIMARY tumours of the orbit are uncommon; the malignant orbital tumours seen by the radiotherapist are usually of secondary origin. Table I lists the material seen at the Royal Marsden Hospital during the period 1933–55. This material is clearly a selected one and cannot be used for assessing the incidence of orbital neoplasms.

TABLE I

ORBITAL TUMOURS SEEN AT THE ROYAL MARSDEN HOSPITAL
1933–55

Primary	Sarcoma of lymphoid tissue	14	
	Rhabdomyosarcoma	7	
	Other sarcomata	6	42
	Lacrimal gland tumours	14	
	Nerve tissue tumours (glioma of optic nerve)	1	
Secondary	From: Intra-ocular tumours	6	
	Neoplasma arising in the paranasal sinuses	100	126
	Metastatic	10	
	From: Generalised lymphoid tissue	10	
Benign tumours and granulomata		22	
	Total	190	

A general account of the natural history of these tumours, the results of radiotherapy and the complications following such treatment, have been published in detail elsewhere (Lederman, 1956). It is the purpose of this paper to furnish an account of the techniques employed in the radiation treatment of orbital tumours at the Royal Marsden Hospital.

The technique of treating an orbital tumour depends mainly on whether the eye is present and whether the tumour is primary or secondary. If a sound eye is present, no effort should be spared in its protection, whereas if the eye has been removed techniques can be simplified and extended in range. With a primary orbital tumour, treatment can be localised to the orbit with consequent limitation in general and local reactions to radiation; whereas in secondary invasion of the orbit, particularly from the paranasal sinuses, the need to irradiate the orbit

complicates to a great extent the technique of treatment of the primary site, and imposes an extra and often severe burden upon the patient.

A. The Technique of Treating an Orbital Tumour with the Sound Eye Present

The factors influencing the technique are as follows:

(1) *General factors*

(a) *The age and general condition of the patient* do not matter greatly provided the patient is neither too senile nor too young and fractious to co-operate in treatment. Occasionally young children may require sedation at the beginning of treatment, but as a rule they quickly learn to co-operate.
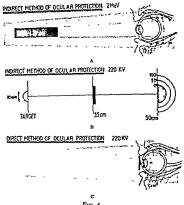
(b) *The state of the eye and orbit.* It may be stated quite categorically that it should never be necessary to remove a normal eye as a preliminary measure to a course of radiotherapy. Under proper conditions the risks of encountering serious ocular damage after radiotherapy are small, and in our series of cases no eye has had to be removed because of damage following external methods of radiation.

Removal of the eye as a preliminary to radiotherapy can be considered if the eye is blind or if a severe exposure or neuroparalytic keratitis is present. Even in these cases, however, removal of the eye is not absolutely necessary and may be avoided should the patient be reluctant to agree to the operation.

Proptosis and chemosis, when present, are always a problem, particularly when due to invasion of the orbit by squamous carcinoma from the paranasal sinuses. In these cases, neoplastic invasion of the orbital bones is accompanied by sepsis, and the presence of a "malignant osteitis" usually precludes a successful outcome to radiation treatment unless the affected bone can be first removed surgically.

Removal of a sound or proptosed eye or exenteration of the orbit in the treatment of antral or ethmoidal cancer is rarely justifiable as a primary measure, for it adds little to the patient's chances of survival. The presence of neoplasm in the orbit is an index of the extensive nature of the disease and if radiotherapy is first applied and fails, then subsequent surgery is no more likely to be successful. In a series of 172 cases of antral and ethmoidal carcinoma

INDIRECT METHOD OF OCULAR PROTECTION 2MeV

A

INDIRECT METHOD OF OCULAR PROTECTION 220 KV

B

DIRECT METHOD OF OCULAR PROTECTION 220KV

C

FIG. 4.
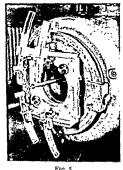
The principles of direct and indirect ocular protection. Note in B the penumbra produced by the interposition of the lead stop, because of this direct protection is preferable when using 220 kV radiation
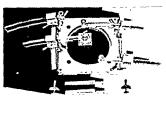
degree of proptosis and chemosis are present but the lids can still be closed, or when the

cornea cannot be properly inspected because of oedema of the lids. In these circumstances a larger area of the proptosed eye has to be protected.

Whichever shield is used it is always placed on the closed upper lid so that its upper margin is in contact with the centre of the upper bony orbital margin, thus allowing for the upward rotation of the globe accompanying closure of the lids (Fig. 3).

(ii) *Indirect protection*

This is necessary when:

(a) 2 MeV radiation is employed,

(b) the eye is severely proptosed and the lids cannot be closed,

(c) the eye is abnormally displaced or immobile owing to an associated ophthalmoplegia,

(d) gross chemosis and oedema of the lids are present, and

(e) there is neoplastic invasion of the conjunctival sac.

The indirect method consists in the insertion in the beam of radiation of an absorbing material, 2 mm lead rubber in the case of 220 kV radiation, or 7·0 cm of lead in the case of 2 MeV radiation, so that the part of the eye to be protected is brought within the shadow produced by the interposed absorbing material. The indirect method can therefore be termed the "shadow-method" (Baclesse, Dollfus, Ennuyer, and Reverdy, 1946), and is for use with

FIG. 5.

FIG 6
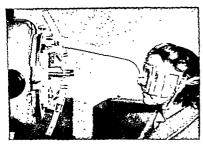
FIG 5. Indirect ocular protection. A general view of the insert holder in position on the face of the 2 MeV generator. The recessed position of the eye shield close to the front shutters is clearly seen.

FIG 6 Indirect ocular protection. The aluminium insert holder with four attachments. The large square shield in place covers 6 × 6 cm at 67 cm F.S.D.

known and its extent can be gauged by clinical and radiological findings.

The basic technique used is to irradiate *en bloc* the whole antrum, ethmoid and orbital cavity on the affected side (Figs. 2 and 3) and to arrange for ocular protection by placing the antero-lateral and lateral fields so that the beams of radiation pass behind both eyes, and by shielding the eye on the affected side from direct irradiation by the anterior field.

In contrast to antral and ethmoidal cancer where nearly half the facial skeleton has to be treated, with post-nasal tumours treatment is directed to the post-nasal space alone, unless evidence of spread outside this region can be demonstrated. If the orbit is involved as shown by proptosis, an anterior field is

Radiotherapy has a sinister reputation among ophthalmologists: it was largely gained in its period of development, and now persists in rather a traditional fashion. Whilst under modern conditions the ocular disasters encountered in the early days of radiotherapy are rarely seen, it would be quite wrong to give the impression that the irradiation of a malignant orbital tumour is without risk. Complete protection of the eye in these circumstances cannot be obtained, nor is it desirable, since tumour tissue may simultaneously be protected: the most one can hope to achieve is to protect from direct radiation the vulnerable ocular tissues forming the anterior segment of the eye, namely, the cornea, iris, ciliary body and lens.
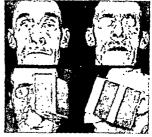
FIG. 2.   FIG. 3.

FIG. 2. Treatment of a maxillo-ethmoidal tumour by supervoltage therapy (2 MeV). Note indirect method of ocular protection (*see* Figs. 5 and 6). A similar treatment set up using two fields is employed for primary orbital tumours.

FIG. 3. Treatment of a maxillo-ethmoidal tumour at 220 kV using a cast. Note direct method of ocular protection.

added over the affected eye with direct or indirect corneal protection and the whole orbit treated, since invasion usually takes place from behind and selective protection of the orbit is unwise.

Ocular protection
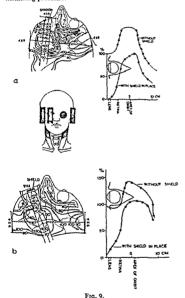
In advocating radiotherapy for the treatment of malignant orbital tumours, one must be ever conscious of the possible risks of damage to the normal eye. If these risks are considerable, then a case can be made out for removing the eye before embarking upon radiotherapy. If, however, the risks can be shown to be small or otherwise worthwhile accepting, then clearly the eye should be left since it is far better for a patient to retain the eye even at the cost of impaired vision than suffer its removal, which is inevitable should a malignant orbital tumour be submitted to surgical treatment.

Ocular protection may be obtained in two ways, directly or indirectly, depending on the degree of proptosis present and the quality of the radiation employed.

(i) Direct protection

This can be obtained most simply when proptosis is not marked and the lids can be firmly closed, by using 200 kV radiation and placing a piece of lead rubber 2 mm thick upon the closed lids over the part of the eye to be protected. In practice two sizes of lead protection are useful.

(a) A 15 mm circle for use where the cornea and intra-ocular structures deep to it are to be protected from direct radiation;

(b) A 3 × 2 cm oval which shields the whole of the front surface of the eye from direct irradiation. This shield is also used when a moderate

shaped as part of a segment and inclined so that it has the X-ray target as centre. There are two of these plates in the device; they are made of aluminium and are fixed to the aluminium base. Several inserts can be in use at the same time. The base is milled to slide over the applicator-holding face of the Royal Marsden Hospital 2 MeV generator to which it is clamped by two brass screws. The taper of each insert was calculated to allow for the approximate size of the focal spot about 5 mm diameter. It was decided that to minimise penumbra the surface of the insert should be generated by a line joining the outer edge of the focal spot to the edge of the area to be protected. This shape was adopted for protected circular regions for which it presented no machining problems."

reliance upon a single anterior field is only occasionally to be recommended, since the lateral field is necessary to deal effectively with the posterior part of the orbit. When using a lateral field neither direct nor indirect protection of the eye on the treated side is advisable for the following reasons:

(1) If the situation of the anterior edge of the lateral field is anterior to the outer bony orbital margin, the attempt to shield the eye directly will inevitably result in partial shielding of neoplasm affecting the antero-medial part of the orbit.

(2) If the anterior edge of the field is at the level of the outer bony orbital margin the plane of the lens and hence the anterior ocular segment is anterior to the direct beam and receives radiation by scatter alone or from the penumbra of the beam.

(3) If the anterior edge of the lateral field is placed 1 cm behind the lateral margin of the bony orbit the beam of radiation may include the posterior part of the globe, but the whole of the anterior segment and most of the posterior segment of the eye are certainly avoided.

Figs. 7, 8 and 9 show the influence of corneal shielding upon the radiation dose distribution within the orbit at 220 kV and 2 MeV for various treatment techniques. The quantity of scattered radiation reaching the "protected" intra-ocular structures at the former voltage is appreciable and may be sufficient to give rise to a cataract in spite of the shielding used.

For the rare retro-ocular tumours, particularly those associated with the optic nerve, an entirely different type of technique is available. This technique, using 220 kV X rays with a light device, depends on the use of a temporal and nasal field, the eye being protected by fixing the gaze away from the incident beam of radiation so that the anterior segment of the eye lies outside the beam. A technique of this kind was originally devised at the Royal Marsden Hospital (Walton, 1952) for the treatment of intra-ocular lesions affecting the posterior segment of the globe and it can in exceptional cases be used for small posterior orbital lesions which are unassociated with marked proptosis and when eye movements are full and the patient can actively co-operate.

FIG. 9.

Depth dose on a ray through the cornea showing the effect of shielding. Carcinoma of the post nasal space.

    a. 2 MeV 67 cm F.S.D., 11 7 mm Cu H.V.L.
    b 220 kV 50 cm F.S.D ; 1·5 mm Cu H.V.L.

(3) Dosage

There is a material variation in the radio-sensitivity both of the various component tissues of the normal eye and the differing orbital neoplasms encountered. Thus the outer eye, comprising the lids, conjunctiva and cornea, tolerates radiation well

Any shielding of the cornea and the underlying intra-ocular tissues inevitably means that there will be shielding or inadequate irradiation of some part of the neoplasm which happens to involve the retro-ocular segment of the orbit. It is for this reason that

a light device only, being impracticable where ordinary closed-ended applicators are employed. The regions of total protection produced on the front surface of the eye in this way are equivalent in area to the direct shields used, although at 220 kV there is an additional peripheral region of partial protection which means that at this voltage "indirect" protection is less satisfactory than "direct".

Description of apparatus

"The difficulties presented by the employment of any method of ocular protection with supervoltage radiation, are due to the high percentage transmission of the radiation through moderation thicknesses of the normal shielding materials. For a reduction of the primary intensity to 1 per cent, a thickness of 7 cm of lead is necessary at 2 MeV. If it is required to protect a small region of say 1·5 to 2·0 cm diameter lying anywhere inside the radiation field defined by the diaphragm system, the inserted lead shield 7 cm long must always lie along some ray through the target whatever
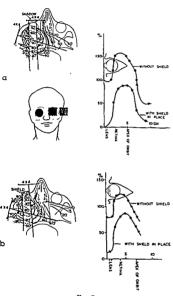
FIG. 7.

Depth dose on a ray through the cornea showing the effect of shielding. Primary orbital tumour.

  a 2 MeV 67 cm F.S.D., 11·7 mm Cu H.V.L.
  b. 220 kV 50 cm F.S.D., 1·5 mm Cu H.V.L.

FIG. 8.

Depth dose on a ray through the cornea showing the effect of shielding. Carcinoma of the antrum.

  a. 2 MeV 67 cm F.S.D., 11·7 mm Cu H.V.L.
  b. 220 kV 50 cm F.S.D., 1·5 mm Cu H·V·L.

A special apparatus for indirect protection (Figs. 5 and 6) has been designed and made at the Royal Marsden Hospital for use with 2 MeV Van de Graaff apparatus. I am indebted to Mr. J. Geilinger, who has designed the apparatus, for the following brief account.

its position in the field. This kinematic problem has been solved in the present insert holder by attaching the shield to one end of a sliding Perspex arc, whose outer surface is of the correct radius, moving through a brass clamp. The Perspex arc terminates in a short length of Perspex tubing through which passes a brass screw to hold the required insert. A degree of freedom perpendicular to the movement of the arc is provided by sliding the brass clamp on a plate

(121)

The absence of the eye simplifies technique of treatment by external radiation and also permits the safe use of radium application or implantation.

(1) Post-operative irradiation of the socket may be given by external radiation or by contact methods. External radiation can be used for massive recurrence in the socket or for routine post-operative treatment after removal of the eye for an intra-ocular tumour showing extra-ocular spread, *i.e.* melanoma or retino-blastoma. The technique is the same as described for orbital tumours using X rays at 220 kV; but the only structure requiring any special protection is the remaining eye. For local recurrence in the socket of very limited extent, a special socket radium applicator (Fig. 10) can be employed, an orbital implant (Fig. 11) undertaken, or contact X rays used.

The place of telecurie therapy using radium or radio-cobalt units at short distances in the treatment of orbital tumours has not been discussed because the problems of protecting both eyes are considerably greater using these instruments than when using high voltage or supervoltage therapy. For this reason the main indication for telecurie therapy is in the treatment of lymph node deposits in the pre-auricular or cervical regions.

Fortunately, lymph nodes are rarely a problem with primary orbital tumours, being more commonly encountered with secondary tumours. Deposits in lymph nodes occurring secondary to an orbital sarcoma should always be treated by radiotherapy. Block dissection is occasionally advocated for cervical lymph node metastases from lacrimal gland carcinomata or more frequently squamous cell deposits secondary to paranasal sinus carcinoma.

SUMMARY

An account is given of the factors governing the techniques of treating orbital tumours, both benign and malignant. A device for protecting the eye when using 2 MeV radiation is illustrated and briefly described and the principles of ocular protection discussed. An account is also given of some simple technical methods of treating orbital tumours after removal of the eye.

REFERENCES

BACLESSE, F., DOLLFUS, M. A., ENNUYER, A., and REVERDY, J., *J. Radiol Electrol.*, 1946, xxvii, 516.
LEDERMAN, M., *Brit. J. Ophthal.*, 1956, xl, 592.
WALTON, R. J., *Brit. J. Radiol.*, 1952, xxv, 9.

and any reactions occurring usually heal without serious or permanent damage. The normal cornea is very tolerant of radiation even when high doses are given—providing sepsis and trauma are avoided. Of the intra-ocular structures the lens alone is highly radiosensitive, a single dose of 600 r, or 1500 r in one month, is almost certain to produce a cataract. Little is known concerning the radiosensitivity of the retina and uveal tract, but under therapeutic irradiation, they rarely seem to suffer damage from radiation.

Secondary orbital invasion from cancer of the paranasal sinuses may prove most obstinate and high doses of 5000–6000 r in 4–6 weeks often prove inadequate, especially with extensive bone involvement. Secondary deposits from other primary tumours or spread from intra-ocular tumours can usually be controlled by doses of the order of 4500 r in one month, and the secondary manifestation of the reticuloses are as sensitive or often more so than the primary lymphoid sarcomata.
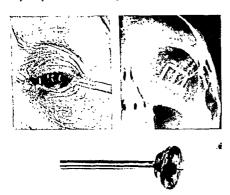
FIG. 10.

FIG. 11.

FIG 10 Perspex radium applicator for post-operative treatment of a socket. The dimensions of the applicator are those of the eyeball and it is loaded with 5 × 5 tubes.

FIG. 11. Orbital radium implant for recurrence in the socket. This technique is contra-indicated if a sound eye be present on the affected side

Tumour sensitivity and, therefore, dosage required varies considerably. Thus the lymphoid sarcomata are radiosensitive and a dose of 2000–3000 r delivered over a month prove adequate for most cases. The rhabdomyosarcomata are very radiosensitive, but in view of their tendency to immediate recurrence, full dosage over a long time period is advisable, i.e. at 220 kV, 5000 r in 6–8 weeks; 2 MeV, 6000 r in 6–8 weeks. The temptation to stop treatment soon after the disappearance of a tumour after a dose of 2000–3000 r should be resisted.

Lacrimal gland tumours treated either pre- or post-operatively also require full dosage, 4000–6000 r in 6 weeks, the lower dosage being employed with 220 kV X rays.

In treating benign lesions, safety must be sought in the use of the lowest possible radiation dosage, i.e. 50–100 r weekly to the granulomata for 4–6 weeks, a single dose of 250–400 r for the angiomata repeated no more than twice in the course of a year or 18 months, and 2000 r in one month for the nerve sheath tumours.

B. The Technique of Treating Orbital Tumours after Removal of the Eye

After removal of the eye or exenteration of the orbit, post-operative treatment may be required as an immediate measure because of doubtful or incomplete surgical removal of all the tumour, or because of frank recurrence in the socket or orbital cavity.

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may be accompanied either by no fluid or by only a small static pleural effusion.

In 11 of the patients under review with primary carcinoma of the breast, ovary, or cervix, or lymphosarcoma small pleural effusions were discovered in addition to the major recurring effusion in either the peritoneal cavity or the contralateral pleural sac needing treatment with radioactive colloidal gold. These small symptomless effusions, in which tumour cells were detected, either remained static or decreased over a period of some months.

It is well recognised that in some patients with malignant effusions considerable quantities of fluid may reaccumulate within a few hours of removal. Studies of the turnover of human serum-albumin labelled with radioactive iodine and of water labelled with tritium (radioactive isotope of hydrogen) have shown that about 100 litres of fluid is transferred across the vascular-peritoneal barrier in twenty-four hours (McKee et al. 1952, Prentice et al. 1952, *British Medical Journal* 1954). It is therefore readily understandable how small deviations from normal in either absorption or secretion could give rise to rapid accumulation of fluid.

Fig. 3—Section of lung and pleura 11 weeks after intrapleural administration of 80 mC of radioactive colloidal gold, showing infiltration by malignant cells (carcinoma of breast) and thickening of pleura due to progressive organisation of fibrinous exudate (× 120).

Mode of Action of Radioactive Colloidal Gold

The following mechanisms are most likely to be involved in inhibiting the formation of fluid (Andrews et al. 1953):

(1) *The production of subendothelial fibrosis* would obliterate regional blood-vessels and lymphatics and inhibit the exudation of fluid therefrom. In some of the present patients coming to necropsy, histological examination showed thickening and fibrosis of the serous membrane previously treated with intracavitary colloidal gold (fig. 3).

(2) *The promotion of adhesions* between the visceral and parietal layers of the treated serous membrane would diminish the surface area responsible for the formation of fluid and partly obliterate the available space in which fluid could collect. At necropsy extensive adhesions are often found in the treated cavity. The formation of adhesions is, however, not essential for the success of intracavitary gold therapy : 2 patients responded well for three or four months, and then ascites again developed, and laparotomy in both cases showed that the peritoneal cavity was free from significant adhesions.

(3) *Direct Effect of Irradiation on Tumour.*—About 90 % of the dose is due to beta radiation, with a half-depth value in tissue of only 0·4 mm ; the gamma radiation amounts to only a small fraction. It is therefore most improbable that these relatively small doses of irradiation produce any

appreciable effect on tumour surfaces so far as the formation of fluid is concerned. In some cases of advanced mammary cancer with pleural effusions and diffuse metastases hormone therapy produces a dramatic response of the metastases in soft tissue and bones, while
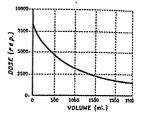
Fig. 4—Volume plotted against surface dose (from 100 mC ¹⁹⁸Au) showing fall-off in surface dose with increasing dilution, the colloidal gold being assumed to remain permanently within fluid.

the pleural effusion increases owing to the retention of fluids caused by œstrogens and androgens. In these cases it can reasonably be assumed that the pleural metastases are responding in a similar dramatic fashion as the other secondary deposits, but that the pleural fluid increases because it is not being secreted by the tumour. Furthermore Bonte et al. (1956) report that the intrapleural administration of nitrogen mustard in cases of recurrent malignant effusions secondary to breast cancer gives results similar to those achieved by treatment with colloidal gold. Except for non-specific general radio-mimetic effects, nitrogen mustard does not possess any selective action on breast cancer ; hence any benefit produced by this therapy is more likely to be due to the production of fibrosis than to any selective effect on malignant cells.

Technique of Administration of Intracavitary Colloidal Gold

Every effort is made to remove all available fluid before the intracavitary instillation of radioactive colloidal gold, because fluid in the treated cavity will reduce the dose-ratio to the serous membrane, if the gold particles remain within the cavity. Fig. 4 illustrates the effects of dilution on the surface dose delivered by 100 mC of colloidal ¹⁹⁸Au. In practice, however, it is well known that particles of colloidal gold are fairly rapidly removed from the intracavitary fluid and deposited in aggregates of various sizes in the subendothelial connective tissue. Hahn (1956) has suggested that a considerable volume of saline solution should be given simultaneously with the colloidal gold because this procedure would accelerate the deposition of gold in the subendothelial tissues.

Patients presenting with large pleural effusions should have the greater part of the fluid aspirated one or two days before the start of treatment with colloidal gold ; if this step is omitted, it will only be possible, at the time of instillation of the colloid, to aspirate three to four pints of fluid before distress, due to mediastinal shift, causes further attempts to remove the remaining fluid to be discontinued.

In some patients with extensive infiltration of the visceral pleura associated with pulmonary collapse the removal of two or three pints of fluid produces a considerable negative intrapleural pressure, which causes such severe pain that further aspiration has to be abandoned.

Peritoneal effusions are best aspirated with a long (15 cm.) cannula having one or two side-openings. By puncturing the peritoneum in the para-umbilical region it is possible to explore most parts of the peritoneal cavity. Where severe symptoms require urgent removal of fluid a polyethylene tube is introduced into the peritoneal cavity, through the cannula before its withdrawal, and colloidal ¹⁹⁸Au can then be instilled through this tube at any convenient time.

RADIOACTIVE COLLOIDAL GOLD IN THE TREATMENT OF PLEURAL AND PERITONEAL EFFUSIONS OF MALIGNANT ORIGIN

REVIEW OF 235 CASES

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Fig. 1.—Cells in ascitic fluid : one cell in metaphase, and another loaded with aggregated particles of radioactive colloidal gold and showing degeneration (Professor Koller's preparation).

and peritoneal effusions of malignant origin (Muller 1950, Walton and Sinclair 1952, Ireton and Ullery 1956, Chang et al. 1957, Hahn 1956). At the Royal Marsden Hospital this form of treatment was begun in 1949 (Smithers 1951), and the clinical and physical aspects of this form of irradiation were discussed by Walton and Sinclair (1952).

235 cases of malignant effusions treated at the Royal Marsden Hospital between March, 1950, and March, 1956, are reviewed here. Samples of fluid from a large number of these patients, both before and after the administration of intracavitary colloidal ^{198}Au, were studied by Prof. R. J. V. Pulvertaft, of the department of pathology, Westminster Hospital, and Prof. P. C. Koller, of the Chester Beatty Research Institute. Using a wide variety of tissue-culture techniques Professor Pulvertaft has so far found it impossible to grow tumour cells found in these malignant effusions. He has kept such cells alive for four to six weeks, but has not been able to get them to multiply in vitro.

Koller (1956) has reported his observations on the cytological analysis of 98 malignant effusions. On the morphology of the cell population he classifies malignant effusions into two types : (1) effusions in which the cells occur singly or in aggregates with well-defined cell outlines (termed free-cell ascites) and (2) effusions in which the cells form masses with indistinct cell boundaries. In the second group polymorphism of chromosomes and mitotic abnormalities are commoner than in free-cell ascites. Koller finds that the cell characteristics are influenced by the volume and rate of formation of fluid and by the intervals between aspirations After the intracavitary application of radioactive colloidal gold the total cell population and the number of dividing cells are much decreased, the range of chromosome polymorphism is greatly reduced, and colloid particles are sometimes observed within tumour cells (fig. 1), which may show eccentric chromosome fragments indicating damage caused by irradiation.

The main object of these cytological investigations was to discover cell characteristics which would indicate the likely effect of intracavitary radioactive colloidal gold on the rate of fluid formation within the cavity concerned. It is generally agreed that not more than half the patients treated derive appreciable benefit from this form of irradiation. It would therefore be most valuable if patients who would derive benefit from treatment with intracavitary colloidal ^{198}Au could be accurately selected on the cytological characteristics observed in a small sample of aspirated fluid.

At present it is, unfortunately, impossible to do this The institution of radioactive colloidal gold consistently reduces the number of tumour cells in an effusion for a time but often does not change the rate of formation of the fluid. Goldie and Hahn (1950) have shown that
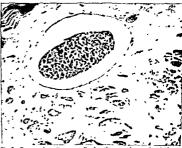
tumour cells are killed by irradiation with radioactive colloidal gold in routine therapeutic concentrations ; but there is no reason to expect that the reduction in the population of free-floating cells would in any way alter the rate of formation of the fluid.

Ætiology of Effusions

The cause of the formation of fluid in malignant disease involving serous membranes is unknown ; several factors may be concerned—e.g., increased transudation (secretion) ; diminished absorption due to obstruction of venous and/or lymphatic vessels (fig. 2) ; and exudation of fluid from raw neoplastic surfaces. It is most unlikely that the last factor is ever responsible for the formation

Fig 2—Group of malignant cells (carcinoma of ovary) within an obstructed and grossly dilated lymphatic vessel in subpleural layer of diaphragm (× 195).

of any appreciable volume of fluid, because patients coming to necropsy who have had to have large quantities of fluid removed at short intervals may not show any extensive neoplastic involvement of the serous membrane in question e.g., in Meigs's syndrome large effusions are absorbed after the removal of an ovarian fibroma which may be quite small Conversely, an extensive involvement of a serous membrane—e.g., subpleural permeation in carcinoma of the lung or of the breast

The cause of the formation of fluid secondary to
malignant disease is still unknown; so is the mode of
action of intracavitary radioactive colloidal gold in
inhibiting the formation of that fluid.

The technique of this treatment and its contra-
indications are described.

I wish to thank Prof D W Smithers, director of the radio-
therapy department, Royal Marsden Hospital, who initiated
this form of treatment at the Royal Marsden Hospital, for
help and encouragement. Professor Pulvertaft and Professor
Koller for investigating specimens of fluid, and Dr. N. G.
Trott and Mrs. G. Dyche, of the physics department of the
hospital, and Miss M Johnson, Miss K Chatterton, and Mr.
J. Gibbs for help in the preparation of this paper.

REFERENCES

Andrews, G. A., Root, S. W., Kniseley, R. M. (1953) Cancer, 6, 294.
Boner, F. J., Storaasli, J. P., Weinberger, A. S. (1956) Radiology.
    67, 63.
British Medical Journal (1954) i, 449
Chang, C. H., Janzen, A. H., Skorneck, A. B., Rosenbaum, P. J.
    (1955) Amer. J. Roentgenol. 77, 486
Goldie, H., Hahn, P. F. (1950) Proc. Soc. exp. Biol., N.Y. 74, 638
Hahn, P. F. (1956) Therapeutic Use of Radioactive Isotopes.
    New York and London
    New York and London
    Ulery, J. C. (1956) Surg. Gynec. Obstet. 103, 137.
Ireton, R. J. (1956) Ann. N.Y. Acad. Sci. 63, 793.
Koller, P. C. (1956) Ann. N.Y. Lamson. B. G., Whipple, G. H (1953)
McKee, F. W. Yuile, C. L.
    J. exp. Med. 95, 161.
Muller, J. H. (1950) Gynecologia, 129, 289.
Prentice, T. C., Siri, W., Joiner, E. E. (1952) Amer. J. Med. 13, 668
Smithers, D. W. (1951) Acta radiol., Stockh. 35, 49.
Walton, R. J., Sinclair, W. K. (1952) Brit. med. Bull. 3, 165

TABLE I—EFFUSIONS IN 235 PATIENTS. RESPONSE TO INTRA-
CAVITARY COLLOIDAL GOLD

Primary site of neoplasm	No. of cases	No. of effusions	Good Inhibition of formation of fluid	Some Inhibition of formation of fluid	No effect	Unknown	Rejected
Breast ..	76	61 Pleural	15	10	9	26	1
		15 Peritoneal	1	..	..	1	..
Ovary ..	71	3 Pleural	1	..	1	1	..
		68 Peritoneal	8	..	5	31	24 (Prophylactic)
Lung Unverified	19	19 Pleural	4	4	4	5	2
	18	2 Pleural	1	..	..	1	..
		16 Peritoneal	6	..	1	9	..
Reticuloses	15	12 Pleural	1	1	3	1	..
		3 Peritoneal	3	..	..	..	..
Colon ..	11	11 Peritoneal	2	..	1	1	4 (Prophylactic)
Miscellaneous *	8	3 Pleural	..	..	..	3	..
		5 Peritoneal	1	..	1	3	..
Uterus body	8	2 Pleural	1	..	..	..	1
		6 Peritoneal	1	..	2	2	..
Stomach ..	7	7 Peritoneal	..	..	7	..	..
Uterus cervix	2	1 Pleural	..	..	1	..	..
		1 Peritoneal	1	..	..	..	..
Total	235		59	16	30	96	34

* Portal cirrhosis, 3; primary adenocarcinoma of liver, 1; cancer of gall-bladder, 1; cancer of pharynx, 1; cancer of lacrimal gland, 1; cancer of urinary bladder, 1.

After the administration of intracavitary colloidal gold the patient is nursed in a special tipping bed. Counting over the peritoneal or the pleural cavity is done routinely, and when this shows any regions of abnormally high or low activity an attempt is made to obtain more uniform distribution by postural tipping. In only 2 cases was it considered necessary to remove the colloidal ¹⁹⁸Au due to local excessive activity; in both patients (one pleural and one peritoneal) about half the administered dose was recovered about twenty-four hours after instillation, and no evidence of any high-dose effects was observed.

Contra-indications

If a reasonable proportion of the fluid estimated clinically and radiographically cannot be aspirated, and if the patient's general condition is very poor and deteriorating, the administration of radioactive gold causes considerable distress and general systemic upset, thereby accelerating and making more unpleasant the terminal phase of the disease without any prospect of affording relief.

Assessment of Results

Table 1 shows the effect produced by intracavitary

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the breast and ovary are the organs most affected. The results are classified as follows:

(1) *Good Inhibition of Formation of Fluid.*—In this group are included patients who, before treatment, required numerous aspirations of large quantities of fluid at short intervals. After the instillation of radioactive colloidal gold the further formation of fluid was completely arrested, or only one or two aspirations were required over a period of many months. In this category are 26 patients who survived longer than a year (table 11).

(2) *Some Inhibition of Formation of Fluid.*—Under this heading are classified patients in whom the rate of accumulation of fluid was retarded; after treatment the fluid was aspirated at longer intervals.

(3) *No Effect.*—In these patients the amounts of fluid and the frequency of aspiration showed no appreciable change after treatment.

TABLE II—26 PATIENTS WHO SURVIVED A YEAR OR LONGER

Site of primary neoplasm	No. cases	Survival (yr.)				
		1	2	3	4	6
Breast	10	4	1	1	2	2
Ovary	8	6	2	..	..	..
Lymphosarcoma (Hodgkin's disease)	4	..	3	1	..	..
Rectum	2	2	..	..	..	..
Lung	1	1	..	..	..	..
Cervix	1	1	..	..	..	..
Unknown	1	1	..	..	..	..

(4) *Unknown.*—In this group are included patients dying within four weeks of treatment; patients who had required only one or two aspirations before treatment and were apparently not forming fluid rapidly or in appreciable quantity; and cases in which no follow-up details were obtained about aspirations.

(5) *Rejected.*—In this category are grouped patients given "prophylactic" colloidal gold therapy.

when an ovarian cyst ruptured during removal or peritoneal seedling deposits, with or without free fluid, were discovered. On these grounds twenty-four patients with ovarian carcinoma were given "prophylactic" gold therapy. Because of wide differences in the stage of the disease, the histology of the lesion, and other forms of additional treatment afforded - e.g., supplementary irradiation with X rays or with radium—it was impossible to assess the value of treatment with radioactive colloidal gold in this group of patients; furthermore, the natural history of malignant disease of the ovary is extremely variable.

The term "seedling deposits" has been used by many surgeons and gynæcologists to include metastatic lesions as large as 2 cm. in diameter. Even in the absence of adhesions it is impossible to irradiate uniformly every aspect of the peritoneal membrane with radioactive colloidal gold, because a layer of colloid 2 mm. thick (fig. 5) is required to surround
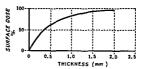
Fig 5—Thickness of fluid layer of colloidal gold plotted against surface dose.

every surface of the membrane, and, in spite of postural tipping, coils of gut lie closely apposed to each other and to the adjacent parietal peritoneum without any intervening layer of fluid. On these grounds it is suggested that uniform irradiation of the peritoneal cavity is best delivered by external means, and that intracavitary gold therapy should only be used as a valuable supplementary form of irradiation which, because of its very superficial action and small integral dose, does not appreciably lower the general systemic tolerance or make it necessary to decrease the dosage delivered by external irradiation.

Conclusion

The administration of intracavitary radioactive colloidal gold is very often the only possible form of treatment

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results of this therapy are by no means dramatic, but a gratifying proportion of patients obtain appreciable benefit in that the formation of fluid is inhibited.

Summary

The results of treating 235 cases of pleural and peritoneal effusions of malignant origin with radioactive colloidal gold are analysed.

activities of around 1000 to 2000 curies. These isotopes are used as sources of high energy radiation and because of their high activity they can be used at treating distances of 50 to 100 cm., thus giving the equivalent of super-voltage x-ray machines (fig. 1). The 'radium bomb' (fig. 2), containing 5 to 10 grammes of radium element, has been in use in a number of radiotherapy units for many years. Such low activity sources require short treating distances (i.e. skin-source distance) of 5 to 8 cm.—because of the inverse square law the intensity of radiation falls off rapidly. This type of apparatus is only suitable for the treatment of super-ficially placed malignant lesions, e.g. larynx and pharynx. Artificially pro-duced radioactive cobalt (^{60}Co) may be used as the radiation source in place of radium element.

Fig. 1.—The telecæsium (^{137}Cs) unit recently installed in the Downs branch of the Royal Marsden Hospital. This is the first apparatus of its kind in clinical use in this country.

RADIOACTIVE ISOTOPES USED FOR IMPLANTATION
(i.e. INTERSTITIAL TECHNIQUES)

In certain sites (e.g. tongue and urinary bladder) circumscribed malignant lesions can often be treated most effectively by local implants of radioactive substances directly into the growth and the immediately adjacent tissues. Such methods permit the irradiation to be confined to a small volume of tissue which can be treated to a relatively high dose level over a period of approximately seven days. If this technique is used it is essential that the radioactive sources should be implanted with absolute accuracy and precision. Until recent years only radium needles or radon seeds were available for this method of treatment and, because of mechanical and technical difficulties, it was often impossible to

Fig. 2.—The radium-bomb irradiating sub-mental region. Note the safe to which the radium is transferred pneumatically when not in use.

RADIOACTIVE ISOTOPES
IN THERAPY

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IN 1948, artificially produced radioactive isotopes first became available for use in medicine in Great Britain. The early optimistic and ill-founded hopes of obtaining a high rate of dramatic cures in malignant disease by means of these isotopes has not been realized. Nevertheless, some of the artificially produced radioactive isotopes have contributed real and fundamental advances in the treatment of many types of malignant disease, and in certain sites (e.g. polycythæmia vera) these isotopes offer the best form of treatment.

The ideal method of treating malignant disease would obviously be by means of a non-toxic chemotherapeutic agent acting specifically, in a lethal manner, on neoplastic cells. In spite of great ingenuity, effort and expense, no such 'aqua vitæ' has yet been discovered. Another approach to this vital problem would be the discovery of any substance possessing the characteristic of being specifically and permanently concentrated to a high degree by neoplastic cells. By artificial means such a substance could be produced in radioactive form and the malignant cells specifically concentrating such a radioactive substance would bring about their own destruction. At present the only such substance in clinical use is radioactive iodine (^{131}I and ^{132}I).

In the treatment of malignant disease artificially produced radioactive isotopes are used in many and varied forms. The selection of any particular isotope technique is in large measure dictated by the exact requirements of the case under consideration. The wide variety of techniques available for the treatment of certain malignant processes by means of radioactive isotopes affords a most flexible, adaptable, essential tool to the armamentarium of the radiotherapist. The clinical applications of the therapeutic uses of isotopes are comparatively new, with the result that modifications and improvement in techniques are constantly taking place.

ARTIFICIALLY PRODUCED RADIOACTIVE ISOTOPES USED FOR EXTERNAL IRRADIATION

The great advantages of high energy irradiation in the 1 to 4 million volt range (i.e. supervoltage therapy), over conventional x-ray therapy machines of around 250 kV, have become fully appreciated in recent years. With such high energy beams skin reactions are minimal, systemic upset very much less, penetration is greater and higher tumour doses can be delivered with greater accuracy. Certain artificially produced isotopes (Cobalt-60, Cæsium-137) emit radiations of high energy and are now obtainable in high

'hormone-dependence' in tumour growth (e.g. breast and prostate) has become fully appreciated. On this basis various methods are used to alter the hormone balance of the patient in the hope of inducing tumour regression. Cases of advanced carcinoma of the breast, which have failed to respond to all forms of routine treatment, may at times derive some benefit from pituitary ablation. This measure can be accomplished by implanting a radioactive source into the pituitary gland (fig. 8) by means of a long needle introduced transnasally through the sphenoidal air sinus and the floor of the pituitary fossa. Pellets of radioactive yttrium oxide (^{90}Y) are most suitable for this technique as this isotope emits only beta-rays.

Fig. 5.—Implantation of bladder tumour, using radioactive tantalum wire.

These rays are highly destructive, but act over a very short distance, thus producing a small zone of necrosis and thereby a local 'radiation hypophysectomy'. High-activity radon seeds were used in some centres, but the additional gamma-rays from this source possess much greater powers of penetration and some patients so treated developed delayed radiation-induced optic nerve damage. Only a small proportion of patients treated by this technique derive any appreciable benefit.

Beta-ray applicators.—Beta-rays penetrate the tissues for only very short distances (1 to 2 millimetres) and this characteristic is utilized in the treatment of certain superficial lesions. Beta-ray therapy is
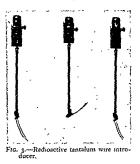
Fig. 6.—Royal Marsden Hospital pattern gun used for the implantation of radioactive gold grains.

often used in the treatment of pathological processes involving the eye (e.g. Mooren's ulcer, superficial punctate keratitis, rosacea keratitis, vascularization of the cornea and allergic conjunctivitis). This type of radiation is most conveniently given by means of a plastic eye-shell (fig. 9), which is coated with radioactive strontium (^{90}Sr). Radioactive phosphorus (^{32}P) can be incorporated in polythene sheets and used in the treatment of certain skin conditions.

obtain accurate disposition of the radioactive sources. Many of the technical difficulties pertaining to interstitial techniques have been overcome by using artificially produced radioactive isotopes.

Radioactive tantalum wire (^{182}Ta), coated with a thin (0.1 mm.) layer of platinum to absorb the beta-rays, can be implanted with a high degree of accuracy, by means of a special intro-ducer (fig. 3), into the muscle wall of the bladder in certain cases of can-cer of the urinary bladder (fig. 4, 5). As these wires are flexible, they accommodate themselves to the vary-ing contours of the bladder wall, and when the period of irradiation has been completed they can easily be removed per urethram. In the treat-ment of certain bladder neoplasms this technique offers many advantages over the older methods employing

FIG. 3.—Radioactive tantalum wire intro-ducer.

radium needles and radon seeds. Because of its flexibility tantalum wire proves a most suitable substance for the preparation of external applicators (moulds) used in the treatment of skin cancers, especially when convex or concave surfaces are involved.

Radioactive cobalt needles are now often used as a substitute for radium needles. Small cobalt beads may be loaded inside thin nylon tubes which are used to implant malignant growths in certain sites; these flexible sources

FIG. 4.—Two radioactive tantalum wires in position in bladder tumour.

can readily be withdrawn from the tissues without having to perform a second 'operation of exposure'. The implantation of platinum-coated gold grains by means of a special gun (Royal Marsden Hospital pattern, fig. 6) offers a most convenient method of applying interstitial irradiation (fig. 7). Magazines, each containing 15 grains, are irradiated in the atomic pile; they can be obtained at short notice and if not used can be re-irradiated. The gun, when primed, will deliver 15 grains (i.e. the contents of one magazine) at one loading. For routine use these grains offer many advantages over radon seeds.

In recent years the importance of

of mossy abnormal pre-malignant mucosa. The proper management of this type of lesion requires treatment to be directed to the whole of the bladder mucosa. This aim can be accomplished by using some form of intravesical irradiation. Such techniques can be administered by introducing radioactive colloidal gold via a catheter or by inserting into the cavity of the bladder a special type of balloon catheter (fig. 12) which is filled with radioactive bromine. By virtue of the proximity of the radiation sources to the bladder wall these techniques give a rapid 'fall-off' in the intensity of irradiation; it is therefore possible to administer a high dose of irradiation to the surface mucosa and at the same time spare the underlying tissues of the bladder wall from excessive damage.

Fig. 9.—Beta-ray applicator: strontium (^{90}Sr) eye-shell.

RADIOACTIVE ISOTOPES IN LIQUID FORM USED SYSTEMICALLY

In this category the isotopes in common use are iodine (^{131}I) and phosphorus (^{32}P). The body cells deal with these artificial radioactive isotopes in exactly the same manner as their naturally occurring non-active counterparts.

Radioactive iodine (^{131}I) in the treatment of thyrotoxicosis.—^{131}I, administered orally, offers a most satisfactory method of treating thyrotoxicosis, and the only factor which prevents this form of treatment from being universally accepted as the method of choice is the hypothetical risk of inducing malignant change in the thyroid gland. Many thousands of patients have now been treated by ^{131}I in various centres all over the world and to date no case of thyroid cancer has been reported. If there is any real risk at all it must be extremely small and after another few years elapse it is almost certain that ^{131}I will become generally accepted

Fig. 10.—Intra-peritoneal insertion of radioactive colloidal gold for treatment of malignant peritoneal effusion.

RADIOACTIVE ISOTOPES USED LOCALLY IN LIQUID FORM

Malignant disease arising primarily in various sites (especially breast, lung and ovary) often involves the pleural or peritoneal membrane, causing the formation of massive effusions of fluid.

These effusions give rise to marked symptoms and require frequent aspirations. If left untreated the patient is soon reduced to a sorry state of misery.

The instillation of radioactive colloidal gold (fig. 10) into the involved serous cavity, following the aspiration of as much fluid as possible, gives rise to appreciable benefit in 30 per cent. to 50 per cent. of patients so treated. A colloid is used in order to prevent the absorption of gold into the blood stream. After the injection of the colloidal gold the patient is tipped into various positions in order to bring all parts of the involved serous cavity into intimate contact with the colloid. Radioactive gold in colloid form emits mainly beta-rays, as well as a small proportion of gamma-radiation. The gold particles are fairly rapidly deposited on the serous membrane where the radiation gives rise to some degree of fibrosis, which is probably mainly responsible for the inhibition of fluid formation. The full benefit from this form of treatment

FIG. 7.—Radioactive gold grain implant of secondary malignant gland.

may take some weeks to manifest itself. Following the intracavitary insertion of radioactive colloidal gold, effusions often re-form quite rapidly. If this postoperative fluid gives rise to appreciable symptoms it can safely be aspirated any time after the fifth day—by then nearly all the radioactivity has been taken up by the serous membrane and any fluid that is aspirated possesses little or no activity.
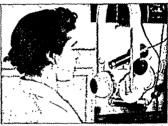
Malignant disease of the urinary bladder may, in a small proportion of cases, first present in the form of multiple small superficial tumours, diffusely spread over all areas of the bladder (fig. 11) with intervening areas

FIG. 8.—X-ray of skull, showing yttrium pellet implant of pituitary gland.

Smooth hyperplastic toxic glands respond to much lower dosages than do nodular glands. Patients with severe toxic symptoms should first be brought under control by bed rest, sedation and antithyroid drugs, because in rare cases ^{131}I can precipitate a thyrotoxic crisis.

Following treatment by means of ^{131}I a small proportion (approximately 10 per cent.) of patients will become hypothyroid; in the majority of these patients this state is only of short duration (i.e. 'temporary post-^{131}I hypo-thyroidism'). The initiation of permanent myxœdema is at times purposely induced by ^{131}I in patients with severe anginal pain due to advanced cardiac disease; this brings about a general lowering of metabolic rate.

Iodine-131 in the treatment of carcinoma of the thyroid gland.—The initial hope that this isotope, by virtue of its highly selective uptake by the thyroid gland, would provide the answer to the treatment of thyroid cancer has not been realized. Generally speaking, only the more differentiated types of growth show worth-while uptake of ^{131}I, and then always to a much smaller degree than nor-mally functioning thyroid tissue. Hence it follows that in order to obtain maximum uptake of ^{131}I in malignant thyroid

FIG. 13.—Estimation of radioactive iodine (^{131}I) uptake by thyroid gland by means of scintillation counter.

tissue as much normal thyroid as possible should be removed, and this may be accomplished by either surgical excision (the more rapid method) or admin-istering a large dose of ^{131}I (ablation dose). About eight to ten weeks later a small tracer (1 mc.) dose of ^{131}I is given and its uptake is determined by external counting (fig. 13, 14). This is followed by a therapeutic dose (100 to 150 mc.) and this procedure is repeated at two- to six-monthly intervals. The uptake of iodine in malignant thyroid tissue may be increased by the administration of antithyroid drugs for a period of two weeks before giving a therapeutic dose; conversely, the administration of any iodine-containing medicine should never be given to such patients as it completely prevents the thyroid tissue from taking up iodine for many months.

Radioactive phosphorus (^{32}P).—Treatment of polycythæmia vera by means of this isotope is now accepted as the method of choice. Patients with this disease have an increased blood volume and red cell mass and blood viscosity. Their numerous and distressing symptoms are due to these hæmo-dynamic abnormalities, which can be verified by blood volume studies using

FIG. 11.—Small superficial papillary tumour (× 25). When the whole bladder mucosa is involved by such lesions intracavitary irradiation offers a suitable method of treatment.

LATEX BAG FOR INTRACAVITARY IRRADIATION OF THE BLADDER

LATEX BAG INFLATED

RUBBER CATHETER

SECTION OF CATHETER

METAL CONNECTION

DRAINAGE OF URINE

FILLING AND EMPTYING OF BAG
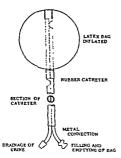
FIG. 12.—Balloon catheter for intracavitary irradiation, using radioactive bromine.

as the best method of treating thyrotoxicosis. At present a very conservative approach is generally recommended and ^{131}I is reserved for specially selected patients: e.g. those over the age of 45 years, those with recurrence of toxic symptoms following partial thyroidectomy (here the results of a second operation are poor and the risk of damage to the recurrent laryngeal nerves higher), and those in poor general condition, as in cases of advanced cardiac failure.

The radioactive iodine is given orally. On entering the blood stream the iodine is taken up by the thyroid gland in a highly selective manner, where it is used to form thyroxine. While concentrated in the gland, highly specific and localized irradiation of the thyroid takes place; this irradiation reduces the activity of the constituent cells and thus brings about a cure of the disease. Various schemes are used for estimating the dose required for any particular patient and the treatment may be given by means of a single dose or repeated smaller fractions. Some improvement in symptoms is often observed after four weeks, but the full benefit of treatment is not evident until three months have elapsed. If no benefit is observed from a single dose at the end of two months, it is unlikely that any further improvement will take place and the dose should be repeated.

SMOKING HABITS OF SCHOOLBOYS*

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THE close connection between smoking tobacco and certain varieties of lung cancer has been conclusively demonstrated. An endeavour should be made to dissuade young people from starting to smoke, and for this purpose it is necessary to know more about their smoking habits.

I summarise here the information kindly supplied by the heads of 141 schools in thirteen counties of England. (5 preparatory schools, 60 secondary schools, 54 grammar schools, 19 boys' public schools, and 3 girls' public schools.)

No further mention need be made of the preparatory schools; for the boys leave at 13–14 years, and the headmasters said that the question of smoking did not arise there. Three of them, however, thought that there boys should be given facts about the harm done by smoking.

SECONDARY SCHOOLS

One headmaster reported that about 80% of his 381 boys have had their first cigarette by the age of 12, and many then stop until the age of 14. Another said that, if the habit starts as early as at 8 years it can be stopped; but at 13 or over it is difficult to overcome without the cooperation of parents. Early smokers are usually boys in "B" and "C" streams, who, being undistinguished scholastically, want to assert themselves otherwise.

Regular Smoking.—In some schools in poor areas, many boys smoke at 9 and 10. One headmaster said that in a poor district it is always possible to find boys who smoke at 11–12, with increasing numbers in each succeeding year. The more "inveterate addicts" are found at 13–15. Another headmaster thought that the proportion of regular smokers is growing, and that at 14 half the boys are confirmed smokers; but in another school not more than 5% were thought to be smoking habitually by the age of 15.

In a survey of 80 boys aged 14–15, 33 did not smoke. Of the smokers, 1 who had started at 15 smoked two cigarettes a week; those who had started at 14 or 13 smoked seven, on the average; those who had started at 12 smoked ten; and those who had started at 11 smoked twenty-three. More detailed figures of another group are shown in table I.

In a school in a semi-rural area, smoking was less than average—certainly less than the headmaster had seen in schools in industrial areas.

Reasons for Smoking.—Mu~~~~~~~ ~~~~~ ~~~~ smoking as a ~~~~~~ tion and of and others f ~~~~~~ ~~~~ ~~~ ~~~ habit from parents ~~~~~ ~~ ~~~ in youth clubs or cadet organisations "~~~y boys at school do part-time work and have money to spend on cigarettes; sometimes parents give boys money with which to buy cigarettes. Many headmasters said that boys smoked with the full consent of their parents, and others called attention to the lack of parental control and bad home conditions in certain areas.

TABLE I—SMOKING AMONG BOYS AGED 15–16

Length of time since first cigarette (yr.)	Cigarettes smoked weekly						
	0	1–5	6–10	11–20	21–30	31	Total
(Non-smokers)	23						23
0–1		12	3			1	16
1		7	2	1			10
2		7		2	1		10
3		3	1	1	1	1	8
4		2		1		1	4
5		1					1
6					1		1
Total	23	31	6	5	3	3	71

Effects of Smoking.—Few headmasters commented on the effects of smoking. One said that more than half the smokers in his school agreed that the habit was harmful but were vague about the effects, the footballers and athletes who smoke or have smoked irregularly were equally divided on whether it affected their wind or speed. In a school which is keen on swimming the headmaster reported that, when teachers noticed that a good swimmer begins to lose form and drop out of competitions, they often find that he has been smoking.

TABLE II—SMOKING-HABITS IN BOYS AGED 15–18

Age	No. of smokers	No. of non-smokers	Average no. of cigarettes smoked per week
15	28	8	14
16	41	5	19
17	21	6	30
18	8	2	19

No outstanding swimmer can maintain his place when he begins to smoke regularly.

Methods of Combating the Habit.—An example from teachers and adults (especially parents) is essential. School rules against smoking are hard to maintain if parents do not cooperate. Before the leaving-age was raised, one headmaster used to cane boys with stained fingers, but such evidence is widely prevalent now. Of 60 headmasters, 54 were in favour of giving boys factual information about the harmful effects of cigarette-smoking. This was done already in some schools, and in one of them there were few smokers. Many headmasters thought that the subject could be introduced during instruction in hygiene, biology, or other science lessons for the boys in upper forms. A few favoured instruction of the younger boys; one thought it could not be started too early. Some pointed out the value of films, charts, and posters. It was suggested that lectures could be given with good effect by visitors to the schools, including doctors, athletes, and Service chiefs. According to one headmaster the strongest deterrent is the unfitness in athletes caused by smoking; according to another it is the cost of the cigarettes. One headmaster suggested that the Ministry of Education could do much more by circularising local education authorities; that the dangers of smoking should be publicised regularly (e.g., on television); that display matter and literature should be distributed to schools; that teachers should be asked to cooperate through their associations; and that blunt warnings should be given from the platform by the head teacher. Another headmaster advised a more positive approach to the problem—i.e., praising physical efficiency rather than decrying smoking.

red blood cells 'labelled' with radioactive chromium (^{51}Cr). These patients are best treated by 'dextran' replacement transfusion and simultaneous venesection, followed by the intravenous injection of radioactive phosphorus (approximately 5 to 7 mc.). The whole treatment can be completed in two to three days. Subsequent injections of ^{32}P are given as required. In this condition the hæmopoietic bone marrow is hyperplastic; it therefore readily takes up ^{32}P and the radiation thereby received reduces its activity.

Lymphosarcoma in its diffuse and generalized form, presenting as massive enlargement of many lymph glands, can be adequately treated by intravenous ^{32}P, which causes a dramatic reduction in the size of the nodes. Radioactive phosphorus is also often used in the treatment of chronic lymphatic and myelogenous leukæmia, by means of repeated small doses.
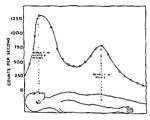
FIG. 14.—Carcinoma of thyroid: Detection of metastatic deposits by profile scan, using a scintillation counter.

CONCLUSION

From this review it is obvious that artificial radioactive isotopes are an essential tool in the management of a large number of malignant processes. This therapeutic weapon is comparatively new and it is certain that it will be directed at many fresh targets in the near future.

Liver Surgery in Relation to Diseases of the Colon and Rectum

PRESIDENT'S ADDRESS

By RONALD W. RAVEN, O.B.E., T.D., F.R.C.S.

THE relationship of the liver to diseases of the colon and rectum is a subject of academic and practical interest to surgeons. In a number of these diseases there are serious changes in the liver and the assessment of its condition is essential. Our knowledge of the behaviour of the liver under various circumstances has been enriched by the latest methods of biophysical and biochemical investigation.

STRUCTURE AND FUNCTION

Since the detailed account by Bradley (1908) of the morphology and development of the liver an extensive literature on this subject has been built up. Electron microscope studies demonstrate the details of this lobulated compound tubular gland and unveil the structure of its polyhedral cells. It shows the protoplasm with its mitochondria, ergasto-

FIG. 1.

FIG. 2.

FIG. 3.
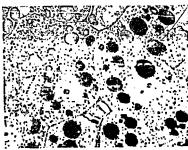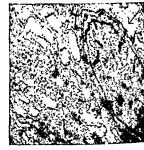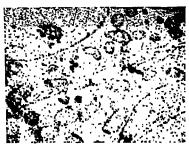
plasm and inclusions of glycogen, fat and protein witnessing to the intensive metabolic activity within the cells. Other features include the large-sized nuclei, the quantity of nucleoprotein contained within the distensible nuclear membrane and the fine network of chromatin granules (Figs. 1, 2, 3).

FIG. 1.—Electron microscope study of rat's liver showing the general arrangement of the cells and a large number of lipid granules in the cytoplasm (arrowed). ×1,250. (Courtesy of Dr Mercer).

FIG. 2.—Electron microscope study of rat's liver showing only a part of two cells; the cell membranes are seen running obliquely down the centre and parts of the two nuclei in the corners (arrowed). Mitochondria with their characteristic [...] ×6,250

a single cell; on the right there is part of a mito-chondrion; a large number of nucleoprotein granules and cytoplasmic membranes are also seen (arrowed). ×37,500 (Courtesy of Dr. Mercer.)

In a group of boys aged 15–18 (table II) half of them were smoking with their parents' consent. At another school many senior boys had given up smoking (table III).

Age when the First Cigarette is Smoked.—One headmaster had seen a boy aged 11 with fingers stained with nicotine. Boys leaving one school were questioned by the headmaster, who found that 5% had started at 14, 10% at 15, 25% at 16, and 5% at 17; 55% had not started. Boys in industrial areas seemed to start earlier than boys in suburban districts. Many able and intelligent boys never smoked; those who did often had a less favourable background than others. Some grammar-school boys learn the habit from boys who have left the secondary modern schools at an earlier age and are at work; others learn in youth clubs. With money from part-time employment some boys have easy access to cigarettes.

TABLE III—SMOKING-HABITS IN BOYS AGED 16–18

Form	Average age (yr., mos.)	No. in form	No. given up smoking	No. decided not to smoke in future
Upper VI	17.11	19	19	19
Lower VI	16.10	26	14	11
VI	15.9	22	15	7
VA	16.9	21	11	6
VBi	16.0	20	10	7
Total		108	69(63%)	50(46%)

Regular Smoking.—One headmaster thought that about 5% smoked regularly before 13, and 25% at 15–16, sometimes with the encouragement or connivance of parents who regard smoking as normal. In one school of 465 boys of varying social background there were probably 45 smokers, of whom 12 were regular. One headmaster pointed out that when boys do smoke, fancy cigarette-cases and fingers heavily stained with nicotine are part of the ritual.

The Type of Boy who Smokes Regularly.—One headmaster was certain that there is a link between smoking and unsatisfactory conduct at school; the only boys he knew as juvenile delinquents were also smokers, and he rarely found the habit among highly intelligent and well-behaved boys. In one school, the only 3 boys who had been convicted (in each case of theft from tobacconists) were smokers; the parents of 2 of them, fearing the effects of repression, had permitted them to smoke.

Smoking Forbidden at School.—In only 5 schools was smoking forbidden. In 1, boys were caned if caught smoking—the youngest offender was aged 12. Several headmasters pointed out that smoking is very common in the teaching profession, and one remarked that boys have only to see the rush for pipes and cigarettes in "break" to realise that their masters do not seem to take much notice of warnings. Masters, like parents, may feel diffident about enforcing the rule. One headmaster had recently given up smoking to convince

a boy in the VIth form that this could be done; but it had little effect on the boy.

Combating the Habit.—44 headmasters were in favour of giving factual information to boys on the ill effects of smoking, 7 were doubtful, and 3 were against it. In one school the doctor saw all the boys at least in their first and fourth years (ages 12 and 15), and spoke to them about the ills of smoking. In another school the Vth-form boys suggested that advice should be given before 13, when many boys start smoking. It may be difficult to convince boys that the danger is personal and not remote in time, and any semblance of tendentious propaganda in its presentation must be avoided. One headmaster who is against this teaching, said that many VIth-form boys are willing to consider objectively problems of modern life, but few base their personal conduct on purely intellectual evidence. Another thought it unnecessary because in his experience smoking has been uncommon among boys for some years; but he was unique in this view. Those who doubted the value of instruction held that because of the example set by adults it is hard to influence the adolescent; more could be done by informal conversation between masters and boys, and the basic safeguard is stricter parental control and a more rigid enforcement of the law about the sale of tobacco. Several headmasters pointed out lectures by outside visitors could carry more conviction than advice from masters who are smokers.

BOYS' PUBLIC SCHOOLS

The replies from the headmasters gave the impression, on the whole, that addiction to smoking is less serious in public schools, though in one, where boys are punished if caught smoking, it is common outside the surveillance of the school. The age at starting is later—often 14–15—and many boys do not smoke at all until they have left school at 18. One headmaster said that on the whole there is less smoking by people in their teens now than ten years ago. In one school the boys are permitted to smoke under certain conditions, and probably not half of them do so. One headmaster said that the habit is formed at home during the holidays.

In one school of 640 boys, of whom 350 were over 16, about 6 smoked habitually before 17 and not many more between 17 and 18. At another school about two-thirds smoked and had begun at 15½, on the average. One headmaster pointed out that few of the boys leaving for universities smoked.

Of 19 headmasters, 12 were in favour of giving factual information to boys, 3 thought it would be of doubtful value, and 1 was not in favour. At one school two-thirds of 70 boys leaving school thought instruction should be given at an early age before smoking is begun.

GIRLS' PUBLIC SCHOOLS

Replies from the headmistresses of 3 public schools suggested that the problem does not exist in these schools. As one said, "it is no longer considered clever to smoke."

Fig. 4.—Liver biopsy showing normal parenchyma in a case of chronic cholecystitis. ×32.
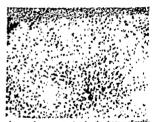
Fig. 6.—Liver biopsy showing the changes of moderately severe biliary cirrhosis—dilatation of biliary sinusoids, peri-lobular fibrosis, lymphocytic infiltration and proliferation of bile ducts. Case of empyema of the gall-bladder. ×32.

Fig. 5.—Liver biopsy showing a minimal degree of fatty infiltration in a case of chronic cholecystitis. ×125.

Fig. 7.—Liver biopsy showing the changes of multi-lobular cirrhosis—patchy fibrosis replacing liver cells with bile duct regeneration. Case of chronic cholecystitis. ×32.

reported a patient who described a detailed report abscesses in the upper and his colleagues (1951) described one patient who developed pylephlebitis and liver abscesses.

(ii) *Inflammatory foci.*—These are scattered tissue. In the normal filtration is lobule is was described by Warren and

(iii) *Interlobular hepatitis.*—Inflammatory round-cell infiltration occurs into the inter-lobular connective tissue with frequent involvement of the adjacent parenchyma where necrosis develops. Later bile duct proliferation and pseudolobulation occur.

(iv) *Hepatic fibrosis.*—A large area of one lobe may be affected without nodular regeneration (Jones *et al.*, 1951).

(v) *Intrahepatic thrombosis.*—Thrombi are found in the hepatic blood vessels; congestion

Its functions are many and varied; they include the metabolism of pigments, carbohydrates, proteins and fats. It is an important excretory organ for bile constituents, toxins, drugs, and antibiotics; other functions are concerned with detoxation, storage, regulation of enzymes, water, salt, hormones and the blood volume.

Assessment of liver function.—This must be done before operation and is required in many colonic and rectal diseases in order to estimate liver damage and safeguard it from further harm. Important work was done by Maclagan (1956) on liver function tests. The tests required for each patient are chosen according to the clinical condition in the light of the clinician's experience.

Liver function tests.—In the interpretation of the results of these tests it must be remembered that a normal range can be found when severe liver damage is present because the parenchyma has a large functional reserve. Approximately one-tenth of the normal parenchyma is sufficient to maintain adequate function (Bollman and Mann, 1931) and this is demonstrated when large primary and metastatic tumours are present.

In the conservative treatment of ulcerative colitis a group of tests is done at intervals and evidence of increasing liver damage influences the decision to carry out surgical treatment. In patients with liver metastases, extensive liver damage as shown by a group of tests may exclude the necessity for an exploratory laparotomy. This group includes a positive urinary urobilinogen, retention of bromsulphthalein and alkaline phosphatase raised above 13 units.

diagnosis can be a decisive factor in lengthening a patient's life provided the appropriate treatment is given. Liver biopsy is performed at laparotomy, by needle aspiration and by peritoneoscopy. I prefer the first when the liver can be inspected and palpated and an adequate wedge of tissue selected and excised (Figs. 4, 5, 6 and 7). Experience is necessary to interpret the histological appearances for they differ from those seen in necropsy specimens. They can differ also in aspiration and surgical biopsies. The following are indications for biopsy:

(1) *To exclude liver disease when liver function tests are abnormal.*—One or more liver function tests can be abnormal when liver disease is absent and this may occur in chronic ulcerative colitis, as shown by this patient:

Male age 38 with severe ulcerative colitis of five years' duration preceded by amœbic dysentery ten years ago. Liver function tests: Serum bilirubin 0 2 mg. %, total serum protein 6 6 grams %, thymol turbidity 5, thymol flocculation 2+, serum colloidal gold 4+, zinc sulphate turbidity 8, ammonium sulphate turbidity 3. Liver biopsy: normal appearance. Operation: total colectomy and anterior resection of rectum with immediate ileo-rectal anastomosis Post-operative liver function tests: Serum bilirubin 0·1 mg %, thymol turbidity 3, thymol flocculation 0, zinc sulphate turbidity 1, serum colloidal gold 0

(2) *To exclude liver disease when the liver is palpable.*—A normal liver is often palpable

1S

Results.—A liver biopsy was considered advisable in 30 of my patients who underwent major abdominal operations. In ⸱⸱ ⸱⸱⸱ ⸱⸱ ⸱ ⸱⸱ of the liver was normal. The abnormalities disco⸱ ⸱ ⸱ ⸱1%), local fibrosis in 3 (10%), fatty degeneration in ⸱ (7%).

THE LIVER IN DISEASES OF THE COLON AND RECTUM

In view of the intimate vascular relationship between the liver, colon and rectum it is surprising that serious effects are not produced more often in the liver by rectal and colonic disease Effective immunity against many liver infections may be provided by its rich blood supply.

Hepatic lesions in chronic ulcerative colitis —These should be recognized so that they may be treated or to safeguard the liver from further adverse effects by surgical treatment of the colitis. In more than 500 patients studied at the Gordon Hospital serious liver lesions were uncommon. Liver function tests were done in about one-half, and approximately one-third of these showed minor deviations from normal, only a small number having severe changes.

The patient's condition gradually deteriorated and on November 16, 1953, a total colectomy and anterior resection of the rectum with an immediate ileo-rectal anastomosis was performed. Liver biopsy showed multilobular fine cirrhosis with some regeneration of the bile canaliculi (Fig 9) The patient has made a good recovery.

(viii) *Amyloid degeneration* secondary to ulcerative colitis is extremely rare.

Hepatic lesions in amœbiasis.—These have a frequency which is determined by the incidence and efficient control of the primary intestinal infection. A diminution has occurred because amœbiasis is now diagnosed earlier and treated more effectively. De Bakey and Ochsner (1951) reported an incidence of liver involvement in 7·6% to 84·4% (average 36·6%) in necropsy cases, and in clinical cases from under 1% to over 25% (average 8·1%) The abscess is often single (70%) and usually affects the right lobe (85%).

The symptomatology may be entirely hepatic without any symptoms of dysentery, Liver function tests, however, are normal. Radiological appearances include elevation of the diaphragm, consolidation of the right lung base and a pleural effusion. Treatment is with emetine hydrochloride or chloroquine diphosphate and if there is no response both drugs are given together. When an abscess forms, aspiration is required in addition to these drugs and antibiotics and open drainage may be necessary when the wall is very fibrotic. When this type of abscess is in the left lobe of the liver a left hepatectomy is preferable to open drainage.

Pyogenic abscess of the liver.—Gangrenous appendicitis was common before antibiotics were available, and then suppurative pylephlebitis was greatly feared. A liver pyogenic abscess is less frequent, however, than an amœbic abscess. Ochsner *et al.* (1938) reviewed 186 cases of liver abscess during 1928–37, of which 139 (74·7%) were amœbic and 47 (25 2%) pyogenic. At present, cholangitis following common bile duct obstruction is the most common cause and the second most frequent is septicæmia.

Other infections in the portal venous area caused hepatic abscesses in 10·2% of the collected cases and 8·4% of the personal cases in the report of Ochsner *et al.* (1938). These included inflammatory lesions in the terminal ileum, cæcum, colon and rectum Kramer and Robinson (1926) reported a case of suppurative diverticulitis with pylephlebitis and liver suppuration; a similar case was reported by Whyte (1906). Other primary infective lesions are bacillary dysentery, infected carcinoma of the colon and rectum, regional enteritis, proctitis and infected hæmorrhoids. Taylor (1949) reported the association of regional enteritis and multiple liver abscesses. Alders (1944) reported a male patient who developed a liver abscess after the ligation of internal hæmorrhoids; the abscess burst into the right anterior subphrenic space which was drained with the recovery of the patient.

METASTATIC CANCER IN THE LIVER

Approximately one-half of patients who die with cancer have liver metastases. This vulnerability of the liver is due to its position in relation to the portal and systemic circulations, its large size and the composition of its cellular contents. It is affected by many varieties of malignancy including carcinoma, especially of the alimentary tract, pancreas, uterus, breast, bronchus, kidney and adrenals; sarcoma; malignant melanoma; argentaffinoma; neuroblastoma; malignant lymphoma and teratoma.

Portal vein dissemination is the main route of spread from the common sites of malignant tumours in the alimentary tract; those in other sites not connected with the portal system may gain entrance by invading viscera already connected, so that tumours in the ovary, uterus, prostate, bladder, kidneys and adrenals can metastasize to the liver.

Segregation of portal blood in the liver.—It has been suggested that blood from different abdominal organs does not mix in the portal vein and has a lobar distribution in that the left lobe receives a disproportionate amount from the superior mesenteric vein. Clinically one lobe of the liver is found sometimes to contain one or more metastases while the other appears free, this is important in relation to partial hepatectomy. We investigated this problem by injecting 0·1 ml. of ^{24}Na with an activity of 50 µc. into a small tributary of the portal vein and the subsequent passage of radioactivity through the right lobe of the liver was recorded by a collimated scintillation counter placed directly over the centre of this lobe. The counter was then moved over the centre of the left lobe and the injection was repeated into a vein adjacent to the previous one. Our studies have not shown any marked asymmetry in the distribution of portal blood between the right and left hepatic lobes. It cannot be postulated, therefore, that a patient with carcinoma in a certain part of the gastro-intestinal tract is more likely to develop metastasis in one lobe of the liver than the other.
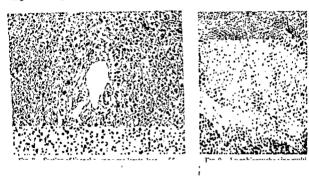
Hepatic artery dissemination.—Metastases reach the liver from primary and secondary tumours in the lung.

Lymphatic dissemination is an uncommon route.

and necrosis occur in adjacent tissues. Thrombosis of the hepatic and portal veins with massive ischæmic necrosis was described by Kleckner et al. (1952).

content of the liver   that fatty infiltration must
be of long duration   al. (1952) failed to find fat
necrosis in severe fa   :8) stated that fatty changes
might cause hepatic insufficiency and cirrhosis. It may be a reversible process.

(vii) *Cirrhosis.*—This is a rare complication. Kimmelstiel et al. (1952) found 11 cases in the literature; Jones et al. (1951) found 3 cases in 91 cases at necropsy; Tumen et al. (1947) reported 5 cases in a series of 151 cases. Kleckner et al. (1952) noted it occurred in young adults with colitis for one to sixteen years. Ross and Swarts (1948) considered that anæmia, marked weight loss and protein deficiency cause hepatic insufficiency which, if uncorrected, progresses to fatty degeneration, and serious, irreversible changes. The nutritional factor may be the most important; deficiencies arise from the defective food intake, faulty absorption and protein loss in the rectal discharge. The patients are also subject to a greater risk of serum hepatitis than the general population. Cirrhosis is usually indicated by jaundice, hepatomegaly alone or with splenomegaly, ascites, abnormal liver function tests and biopsy. In the Gordon Hospital series there is only one patient with typical cirrhosis who is under my care:

Female, aged 40, with a history of ulcerative colitis for two years treated conservatively, with tenderness over the pelvic colon and hepatomegaly. Investigations including rectal biopsy, confirmed ulcerative colitis. Patient was treated conservatively for several months and liver function tests were done at intervals with these results:

	Pre-operative			Post-operative	
	12.1 53	10 6.53	6 11.53	24.3.54	8.10.56
Serum bilirubin	0·3 mg %	1·1 mg.%	0 8 mg %	0 8 mg.%	0·5 mg %
Serum alkaline phosphatase	72 units	58 units	56 units	50 units	27 units
Thymol turbidity	3	6	3	5	2
Thymol flocculation	0	2	1	2	0
Gold flocculation	0	3	2	1	1
Zinc sulphate turbidity	4	7	8	5	3
			Total protein	8 11	7·5
				gram%	gram%

Summary of statistics.—In a series of 1,000 cases of carcinoma of the stomach, colon and rectum, the incidence of hepatic metastases was 222 cases (22·2%). In the total series a partial hepatectomy was considered possible in 41 cases (4·1%). In the series of 222 cases with hepatic metastases, 41 cases (18·5%) are considered suitable in retrospect for a partial hepatectomy; the operation was carried out in 6 cases (2·7%).

Scheme of treatment of metastatic liver carcinoma.—The management of the following groups of patients is described.

(1) *Metastatic liver cancer from a silent primary tumour.*—The liver symptoms and signs dominate the clinical picture. An exploratory laparotomy and liver biopsy confirm the nature of the hepatomegaly.

(2) *Silent liver metastasis.*—This is a frequent finding when operating for a primary carcinoma of the colon or rectum.

(a) *The primary tumour is operable.* When there is diffuse liver involvement the abdomen is closed for the patient's life expectancy is short and palliative treatment is given When considerable liver parenchyma remains and its function is satisfactory the organ containing the primary tumour is removed. This may retard the progress of the liver disease. When liver metastases are localized in one lobe a right or left hepatectomy is done four weeks after the primary disease is removed.

(b) *The primary tumour is inoperable.* Palliative treatment is given.

(3) *Delayed liver metastases after excision of the primary tumour.*—(a) *There is no local recurrence.* When clinical examination indicates that one lobe of the liver is affected it is confirmed by an exploratory laparotomy and if there are no other demonstrable metastases or recurrences a left or right hepatectomy is done.

(b) *Local recurrence is present.* Palliative treatment is given.

Results of hepatectomy operations for secondary cancer

Left hepatectomy.—A survey of the cases reported, including my own, revealed the following results. A left hepatectomy was performed in 16 patients; 7 patients were alive and well 4, 9, 11, 12, 13, 18 and 20 months later; 1 patient was alive with disease 5 months later; 5 patients died 12, 18, 24, 24 and 24 months later; 3 patients were lost sight of. A local excision of the metastasis was performed in 7 patients; 3 patients were alive and well 6, 8 and 48 months later, 1 was alive with disease 15 months later; 3 patients died 4, 7 and 8 months later.

Right hepatectomy.—A survey of the cases reported, including personal communications and my own, gave the following results. A right hepatectomy was performed in 11 patients; 4 patients were alive and well 12, 18, 18 and 18 months later; 1 patient was alive with disease 6 months later; 2 patients died 4 and 24 months later; 4 patients were lost sight of.

SWELLINGS OF THE LIVER WHICH SIMULATE MALIGNANT TUMOURS

Liver swellings occur which resemble malignant tumours and a wrong diagnosis may be made. Surgeons are familiar with "nodules" in the liver which may or may not be metastases. In these cases further investigation should be made. A biopsy of the "nodule" is performed, an operable primary tumour is excised and partial hepatectomy considered later. When a biopsy is not possible and the primary tumour is operable the operation designed to cure the primary disease is performed. The following swellings are considered in this connection.

Liver anomalies.—A good account of these was given by Cullen (1925). A hepatic lobe such as Riedel's may increase in size forming a tongue-like projection from the right lobe which reaches into the right iliac fossa. It is more frequent in females. It may cause intestinal occlusion, as in a case described by Cullen when the hepatic flexure of the colon became adherent to the liver and kinked. Numerous furrows may occur in the liver substance forming intervening lobules which are sometimes pedunculated. Multiple lobes, sessile or pedunculated, may be present in the postero-inferior aspect of the liver.

Liver cysts of various types occur; they include blood vessel cyst, ciliated epithelial cyst, lymphatic cyst and retention cyst.

Hydatid cyst.—According to Dew (1955), 75% of hydatid cysts occur in the liver; the right lobe is affected four times more often than the left. There are two varieties, the first is a unilocular cyst which slowly increases in size, the wall may become fibrosed or calcified and sometimes the cyst is infected and filled with pus. In the second the outer cyst wall is absent and the inner germinative and middle laminated layers invade the liver *like* a malignant tumour.

Spread by direct extension of a carcinoma of the hepatic and splenic flexures, transverse colon and other neighbouring sites is common. The primary tumour becomes adherent to Glisson's capsule, an inflammatory reaction develops and malignant infiltration occurs across this adherent area. Liver necrosis then takes place and severe hæmorrhage occurs into the peritoneal cavity, lumen of the stomach or colon and the patient presents as an emergency.

Spread within the liver.—The vascularity of the liver favours intrahepatic spread. Invasion of the right or left main branches of the portal vein causes metastases in the ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ a wide segment is affected; invasion of ⋯ ⋯ ⋯ ⋯ One parent metastasis can therefore

Spread from the liver.—Metastases invade the efferent hepatic veins entering the inferior vena cava and are carried to the lungs. The inferior vena cava may be directly invaded (Willis, 1952). The liver acts as an intermediary for pulmonary metastases from primary tumours in the portal venous area. Willis (1952) found 86 of these tumours with hepatic metastases had also produced secondaries in the lungs in 27; in 88 tumours without hepatic metastases, lung metastases were present in only 8.

Incidence and operability of hepatic metastases from carcinoma of the stomach, colon and rectum.—An analysis was made of the relevant details in the case-records of 1,000 patients ⋯ ·ual ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ I.

TABLE I.—INCIDENCE OF HEPATIC METASTASES IN CARCINOMA OF STOMACH, COLON AND RECTUM

Site of primary carcinoma	Clinical assessment		Visual assessment—operation or necropsy	
	Total No. of cases	No. with hepatic metastases	Total No. of cases	No. with hepatic metastases
Stomach	84	27 (32·1%)	276	63 (22·8%)
Colon	37	12 (32·4%)	135	33 (24·4%)
Rectum	102	26 (25·5%)	366	61 (16 6%)
	223	65 (29·1%)	777	157 (20 2%)

The operability was assessed in 157 of the 222 cases with hepatic metastases; operation or necropsy was not done in the remaining 65 cases (Table II).

TABLE II.—ASSESSMENT OF OPERABILITY OF HEPATIC METASTASES IN CARCINOMA OF THE STOMACH, COLON AND RECTUM

Site of primary carcinoma	Total No. of cases	No. of possible cases for partial hepatectomy	No. of cases unsuitable for partial hepatectomy
Stomach	63	14 (22·2%)	49 (77·8%)
Colon	33	10 (30 3%)	23 (69·7%)
Rectum	61	17 (27·9%)	44 (72·1%)
	157	41 (26·1%)	116 (73·9%)

In the series of 157 cases assessed visually for hepatic metastases, 116 cases (73·9%) were considered unsuitable for partial hepatectomy due to extensive liver involvement or other inoperable metastases. This leaves 41 cases (26·1%) with hepatic metastases considered operable for further analysis (Table III).

TABLE III.—DETAILS OF HEPATIC METASTASES FOR WHICH PARTIAL HEPATECTOMY WAS POSSIBLE

Site of hepatic metastases	Total No. of cases 41
Left lobe Partial hepatectomy performed	5 (12·2%)
Right lobe. Partial hepatectomy performed	1 (2·4%)
Solitary metastasis in right lobe	14 (34·1%)
Two metastases in right lobe	2 (4·9%)
Multiple metastases in right lobe	1 (2·4%)
Direct invasion of left lobe	4 (9 8%)
Solitary metastasis in left lobe..	3 (7·3%)
Solitary metastasis—site not stated	4 (9 8%)
Two metastases—site not stated	7 (17·1%)

Clinical groups.—(1) In some patients the tumour is an incidental finding, as in the appendix.

(2) In other patients the symptoms are abdominal or rectal, depending on the site of the primary tumour.

(3) Metastases usually occur in the regional lymph nodes and liver and the general effects are seen of 5-hydroxytryptamine secreted by the hepatic metastases which passes into the blood without being converted by the liver into the inactive metabolite 5-hydroxyindole acetic acid. The release of large amounts in the body produces effects resembling those of histamine release including a fall in blood pressure, dyspnœa and flushing of the skin of the neck.

The following case record demonstrates the value of colonic and hepatic surgery in the treatment of this syndrome. A patient had a carcinoma of the cardia and lower

metastases were excised by a partial enterectomy, right hemicolectomy and partial right hepatectomy (Fig. 10).

Fig 10 —Section of liver showing a metastasis of argentaffinoma. ×56.

HEPATECTOMY

History—The development of hepatectomy operations during the nineteenth and twentieth centuries is divided into two periods.

First period.—Segmental resections of the liver were performed with intrahepatic ligation of blood vessels, and considerable ingenuity can be discerned in the technique employed. The end-results of the *nineteenth century* were generally favourable for benign tumours, but much less satisfactory for malignant tumours Local recurrence or generalized metastases occurred frequently several months after operation.

Second period—Lobectomy operations were performed with extra-hepatic ligation of the main blood vessels and lobal duct (Raven 1949) During the first forty years of the twentieth century

DEFINITIONS OF PARTIAL HEPATECTOMY

Wedge resection.—A lesion at, or near, the anterior border of the liver is resected by two angular incisions. *Elliptical resection.*—A lesion superficial in the substance of the liver is resected by an encircling incision. *Transection.*—Part of a lobe is excised by an antero-posterior incision through its substance. *Left hepatectomy.*—A left subtotal or total lobectomy is performed; in the latter operation the caudate lobe is included. *Right hepatectomy.*—The right lobe is removed.

ere reported by Comfort *et al* (1952); in lobe was involved. In about half the

Certain chronic infective lesions can be mistaken for metastatic tumours:

Tuberculosis.—Randolph (1930) reported an autopsy on a patient who died after an operation for uterine carcinoma and found many discrete caseous nodules of inactive tuberculosis in the liver which were confirmed histologically. Miliary tubercles occur in the liver in acute miliary tuberculosis and in chronic tuberculosis of lung and bone. Wolf and Flory (1945) reported a case of ileo-cæcal tuberculosis with liver involvement. A tuberculoma is sometimes found forming a caseating swelling, the lesion may be multiple, secondary to pulmonary or abdominal tuberculosis. The swelling may become large and resembles a necrotic tumour or gumma.

Syphilis.—Gumma may be single or multiple in the liver, which is usually not enlarged unless chronic congestion or cirrhosis is associated. Liver nodules may press on the inferior vena cava and the openings of the hepatic veins into it causing ascites and enlarged collateral venous plexuses. Spontaneous healing is frequent leaving marked scarring and nodularity of the liver.

Actinomycosis of the liver is usually secondary to actinomycosis of another organ and the infection reaches it by direct extension from the stomach, duodenum or transverse colon, or through the portal vein from the appendix, cæcum, colon or ano-rectal region.

Benign tumours may be mistaken for metastatic carcinoma:

Hæmangiomata are found more often in the liver than in any other abdominal organ. They are usually small and found in the left lobe; when multiple they cause nodularity of the liver. They are red-purple, rarely protrude above the surface and are compressible unless calcified.

Adenoma is not rare; it may be asymptomatic and discovered at laparotomy, or it may cause an upper abdominal swelling which presses on, or displaces, neighbouring organs and structures. Its consistence varies from soft to hard and it may be single or multiple (Schrager, 1937).

Connective tissue tumours comprise fibroma, lipoma, leiomyoma and myxoma; they are usually small.

Malignant lymphoma often affects the liver; the extent and nature varying with the type of lymphoma. Sometimes discrete nodules are found which simulate metastatic carcinoma, especially in lymphadenoma and reticulum-cell sarcoma.

Nodules of regenerative hyperplasia of the liver were reviewed by Begg and Berry (1953) when they collected 29 cases from the literature and added 4 more. The nodules were pedunculated (19 cases) or clearly delineated from normal tissue (14 cases); the majority had a nodular surface. Histological examination revealed a pattern usually associated with post-necrotic cirrhosis or subacute hepatitis.

right lobe.

Biopsy of liver "nodule" is frequently necessary to determine its true nature. When the primary tumour is in the pelvic colon or rectum and the abdomen is opened through an infra-umbilical incision visualization of the liver is not possible. In these cases an aspiration-biopsy is done by the method of Pack and Brasfield (1955); the surgeon's right hand is introduced through the wound to identify the "nodule" in the liver so that an assistant can introduce an aspiration needle through the parietes which is then guided by the surgeon into the "nodule".

THE MALIGNANT ARGENTAFFINOMA SYNDROME

Cassidy (1930) described an association of symptoms and signs in a young male patient with an abdominal tumour and liver metastases who experienced a peculiar flushing of the skin of his face and developed pulmonary stenosis.

proteins. Tagnon *et al.* (1948) carried out liver function tests in 20 patients immediately after an extra-abdominal operation and found a significant increase in bromsulphthalein retention in 14. The effects of giving ether and Pentothal-sodium were not important. Geller and Tagnon (1950) investigated changes in the tests in 33 patients after major abdominal operations; in 17 of 22 there were abnormalities in the bromsulphthalein test; in 14 of 22 in the cephalin flocculation test, and 24 of 33 patients had abnormally high serum bilirubin for several days. There was an increased production of bilirubin and post-operative impairment of liver function with blood transfusion. Keeton *et al.* (1948) found the maximum excretion of urinary urobilinogen occurred on the third day after herniorrhaphy and cholecystectomy which returned to normal by the sixth day.

CAUSES OF LIVER DAMAGE IN OPERATIONS

These include trauma, shock, anæsthesia and inanition. Anoxia in anæsthesia or shock causes parenchymal damage. French *et al.* (1952) observed no difference between the effects of ether and cyclopropane; Geller and Tagnon (1950) found no changes in the liver function tests with nitrous-oxide gas, ether and oxygen, spinal anæsthesia alone and combined with Pentothal Sodium. Keeton *et al.* (1948) found that post-operative inanition affected liver function and a high caloric diet containing 2·6 grams protein per kg. resulted in a positive nitrogen balance.

The effects of liver abnormalities can be greatly increased by even minor operations and may cause death. For example hepatic coma may develop in twenty-four to forty-eight hours after a paracentesis. This procedure is usually necessary to relieve dyspnœa or severe abdominal distension, but it must be done with caution; when severe liver damage is present the operation is done in stages. Severe gastro-intestinal hæmorrhage is an important cause of hepatic coma; the retained blood may release ammonia. The hæmorrhage and shock are controlled and when practicable the blood is removed from the colon by an enema.

Other precautionary treatment with liver damage.—Bed-rest is essential when the liver is tender, jaundice is present and liver function tests very abnormal. Protein deficiencies are rectified as much as possible. Avoid acute infections, maintain the urinary output and do not overload with fluid when oliguria is present and not associated with dehydration. Caution is n￼
hypnotics.￼
pethidine ca￼
barbital an￼
chloral hydr￼
be prevented and the electrolyte and water balances maintained. The diet should be rich in protein, such as meat and fish, and 150 grams are taken with 350 grams carbohydrate, and 70 grams of fat. Vitamins and liver extract are included but salt is excluded when ascites or œdema is present.

LIVER REGENERATION FOLLOWING HEPATECTOMY

The liver possesses good power of regeneration; Schroeder (1906) quoted Ponfick who ￼

surgically and regeneration occurs promptly and almost completely.

ACKNOWLEDGMENTS

REFERENCES

ALDERS, N. (1944) *Lancet*, i, 151.

Combined operations.—(1) *In* 1-*stage·* hepato-gastrectomy; hepato-œsophago-gastrectomy; hepato-cholecystectomy; right hepato-hemicolectomy. (2) *In* 2-*stages:* right or left hepatectomy following a left hemicolectomy or an abdomino-perineal excision of the rectum.

PRINCIPLES OF TECHNIQUE

Left hepatectomy (Raven, 1949).—The abdomen is opened through a transverse incision made half-way between the umbilicus and ensiform cartilage which commences 5 cm. to the right of the mid-line and ends at the left costal margin; a second incision is made at

now retracted to the right and the left branch of the portal vein, left hepatic duct and the left branch of the hepatic artery are ligated and divided.

There are usually three main hepatic veins from the posterior aspect of the liver which join the inferior vena cava in the posterior hepatic groove. One vein passes from the right lobe and another from the left lobe, which open obliquely into the inferior vena cava; the third vein is from the middle of the liver and spigelian lobe and it pursues a straight course to join the main vein. The left and middle veins are ligated and divided.

To remove the left lobe, an oblique antero-posterior incision is made with the diathermy knife from the attachment of the round ligament to end posteriorly immediately to the

region of the divided liver.

Right hepatectomy (Raven, 1947; Lortat-Jacob and Robert, 1952; Pack and Baker, 1953).—The same technical principles are applied to this operation. The abdomen is explored through a right paramedian incision which is then extended into the pleural cavity through the right eighth intercostal space. The right leaf of the diaphragm is divided radially towards the inferior vena cava. The right lobe of the liver is mobilized by ligating and dividing the round ligament, dividing the longitudinal and right coronary ligaments. The cystic duct is then ligated and divided; the right hepatic duct and the right branch of the hepatic artery are ligated and divided. The right branch of the portal

the main right hepatic vein joins the inferior vena cava.

Any bleeding vessels and small bile ducts in the divided left lobe are treated. A raw bare area is peritonealized using the c
the hepatic artery, common hepatic c
then sutured and the right pleural cavity is closed with underwater drainage, a soft rubber tube is placed in position to drain the region of the divided liver and the abdominal wound is closed.

EFFECTS ON THE LIVER OF OPERATIONS

Experimental work has shown morphological and biochemical changes in hepatic cells. Cole and Leuchtenberger (1956) compared the histological appearances in dogs pre- and post-operatively. In the pre-operative liver the chromatin in the nuclei was finely granular and evenly distributed, but in the post-operative liver it was coarse, coalesced, distributed irregularly, and large rarefied areas were present. The nucleoli after operation became larger with irregular borders. The changes were most obvious three to six hours after operations and no appreciable diff... three or four days later, and the These workers also found an increase following operation, but no change The interpretation of these results is not yet clear; they may indicate increased protein synthesis by the liver or the presence

operative liver, greater increases were found in those given blood transfusions. No changes occurred in the flocculation tests, and in 2 patients a mild fall occurred in the total serum

THE TREATMENT OF CARCINOMA OF THE OESOPHAGUS*

by

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INTRODUCTION

CANCER OF THE oesophagus presents a peculiarly difficult set of treatment problems. These tumours occur in a thin-walled, deeply placed tube liable to fatal perforation. The first symptom is usually dysphagia which comes on comparatively late in the development of the disease, there is often considerable delay in reaching hospital, and these patients when first seen by a surgeon or radiotherapist tend to be starved, wasted, old and ill. Malignant tumours may spread widely up and down the length of the oesophagus, making it difficult to define their precise limits; many of them are poorly differentiated and have already metastasised by the time that the patient comes for treatment. When I last published a paper on the treatment of carcinoma of the oesophagus thirteen years ago (Smithers, 1943), I was able to find only one patient reported in the literature who had survived more than five years following resection. This was the famous but quite unusual case of Torek's, where the patient had had symptoms for as long as nine months before treatment but was still found to have a small localised tumour at the time of operation. Several reports of patients surviving five years or more after radiotherapy were then to be found but they were scarce and some were unreliable.

During the last ten to fifteen years, a great effort has been made to improve this position both by surgeons and radiotherapists, and it is my purpose here to review that effort, to try to see what has been achieved and to assess its present value. There is no doubt that a new opportunity for surgery at this site has been created by modern anaesthesia, antibiotics and improved post-operative care and that this opportunity has been seized with enthusiasm by many and with great skill by a few. We should judge the value of this treatment by the best that has been achieved. In 1943 I gave an account of the first radiotherapeutic attempt to use modern three-dimensional dose distribution estimations combined with accurate beam direction to see if this might improve the results obtained. Radium bougie treatment had been shown to increase the perforation rate though it had an occasional success to its credit; supervoltage irradiation was not available; and although radiotherapy had a good deal more to show to its credit in dealing with cancer of the oesophagus than surgery, it was then still making its effort with out of date methods and inadequate apparatus. It is now clear that radiotherapy has at last a real opportunity to show what it can do for patients with cancer at this site through the

BRADLEY, O. C. (1908) *J. Anat., Lond.*, 43, 1.

CASSIDY, M. A. (1930) *Proc. R. Soc. Med*, 24, 139.

COLE, J. W., and LEUCHTENBERGER, C. (1956) *Surg. Gynec. Obstet.*, 102, 702.

COMFORT, M. W., GRAY, H. K., DAHLIN, D. C., and WHITESELL, F. B. (1952) *Gastroenterology*, 20, 60.

CULLEN, T. S. (1925) *Arch. Surg., Chicago*, 11, 718.

DE BAKEY, M. E., and OCHSNER, A. (1951) *Surg. Gynec. Obstet.*, 92, 209.

DEW, H. R. (1955) In: Abdominal Operations. Ed. R. Maingot. New York; p. 869.

ELSOM, K. A., and FERGUSON, L. K. (1941) *Amer. J. med. Sci.*, 59, 202.

FRENCH, A. B., BARSS, T. P., FAIRLIE, C. S., BENGLE, A. L., JONES, C. M., LINTON, R. R., and BEECHER, H. K. (1952) *Ann. Surg.*, 135, 145.

GELLER, W., and TAGNON, H. J. (1950) *Arch. intern. Med.*, 86, 908.

HIMSWORTH, H. P. (1950) The Liver and Its Diseases. 2nd edit. Oxford.

—— , and GLYNN, L. E. (1944-5) *Clin. Sci.*, 5, 93.

JONES, G. W., BAGGENSTOSS, A. H., and BARGEN, J. A. (1951) *Amer. J. med. Sci.*, 221, 279.

KEETON, R. W., COLE, W. H., CALLOWAY, N., GLICKMAN, N., MITCHELL, H. H., DYNIEWICZ, J., and HOWES, D. (1948) *Ann. intern. Med.*, 28, 521.

KIMMELSTIEL, P, LARGE, H. L, and VERNER, H. D. (1952) *Amer. J. Path.*, 28, 259.

KINNEY, T. D, and FERREBEE, J. W. (1948) *Arch. Path.*, 45, 41.

KLECKNER, M. S, STAUFFER, M. H., BARGEN, J. A, and DOCKERTY, M. B. (1952) *Gastroenterology*, 22, 13.

KRAMER, S. E., and ROBINSON, W. (1926) *Surg. Gynec. Obstet.*, 42, 540.

—— —— .... (1922) *Med. Cl... N. Amer* 16, 1427.

.... Ed. Leon Schiff. London; p. 125.

—— , and BRASFIELD, R. D. (1955) *Amer. J. Surg.*, 90, 704.

PETTINARI, V. (1947) *Minerva med*, Roma, 1, 134.

PICKRELL, K. L., and CLAY, R. C. (1944) *Arch. Surg., Chicago.*, 48, 267.

POLLARD, H. M., and BLOCK, M. (1948) *Arch. intern. Med.*, 82, 159.

RANDOLPH, B. M. (1930) *Amer. Rev Tuberc.*, 22, 593.

RAVEN, R. W. (1947) *Brit. med J.*, II, 249.

—— (1949) *Brit J. Surg.*, 36, 397.

—— (1950) *Proc. R. Soc. Med.*, 43, 675.

ROSS, J R., and SWARTS, J. M. (1948) *Gastroenterology*, 10, 81.

SCHRAGER, V. L. (1937) *Amer. Surg.*, 105, 33.

SCHROEDER, W. E. (1906) *Surg. Gynec. Obstet*, 2, 52.

TAGNON, H J., ROBBINS, G. F., and NICHOLS, M. P. (1948) *New Engl. J. Med.*, 238, 555.

TAYLOR, F. W. (1949) *Amer. J. Med.*, 7, 838.

THORSON, A, BJÖRCK, G, BJÖRKMAN, G, and WALDENSTRÖM, J. (1954) *Amer. Heart J.*, ..., ...

TREUSCH, J. V. (1952) *Gastroenterology*, 20, 166.

TUMEN, H. J., MONAGHAN, J. F., and JOBB, E. (1947) *Ann. intern. Med.*, 26, 555.

WARREN, S., and SOMMERS, S. C. (1949) *Amer. J. Path.*, 25, 657.

WHYTE, J. M. (1906) *Scot. med. surg. J.*, 18, 120.

WILLIS, R. A. (1952) The Spread of Tumours in the Human Body. London.

WOLF, G. A, and FLORY, C. M. (1945) *Amer. Rev. Tuberc.*, 51, 555.

oesophageal cancer reported here. The number of patients with adeno-carcinomas included is twenty-six : these are divided into upper third, one ; middle third, four ; and lower third, twenty-one. Each case of lower third adenocarcinoma has been reviewed and only included when good evidence for primary oesophageal origin existed. The individual
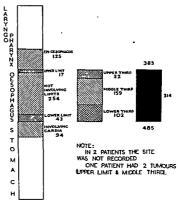
Fig 1. Selection for diagnosis of cancer of the oesophagus from all those new patients first seen at the Royal Marsden Hospital, 1936-1951, with adjacent tumours.

reasons for this selection have already been given (Smithers, 1956). There are no known special methods of selection of the patients with oesophageal cancer coming to the hospital Most of these are sent to individual members of the staff by general practitioners or come from other hospitals for radiotherapy (Table I) The fact that it is a special hospital for malignant disease no doubt affects the selection, but it is difficult with tumours at this site to see how the material differs from that seen at a general hospital.

The number of patients seen in each of the four four-year periods covered by this report, and the proportion of the total treated by surgery and by radiotherapy are shown in Table II. It has been our policy to try

use of supervoltage X-rays and telecurie therapy units combined with accurate multiple-field or rotation techniques Again we should judge the value of the effort by the best that is being achieved with these new methods

The number of five-year survivors following both surgery and radiotherapy—though still a pathetically small proportion of the total treated —has shown a definite increase I propose to present to you an account of the patients seen at the Royal Marsden Hospital over the period covered by the introduction of modern surgery and radiotherapy for oesophageal tumours—that is, from 1936 to 1951. I shall then try to compare these with the results obtained elsewhere to see if I can give some indication of where these changes are leading us and what we may expect from them as they come into more general use in the future.

Royal Marsden Hospital results

The upper and lower limits of the oesophagus are not well defined , at the upper limit difficulty exists in separating tumours of the lower laryngopharynx from those of the upper cervical oesophagus—indeed, it may at times be impossible to do so Tumours of the lower end may arise astride the cardia, a strict division between oesophageal and stomach tumours by histological type alone is inaccurate, and different views about the origin of those adenocarcinomas which are confined to the lower oesophagus lead to differences in classification (Smithers, 1956). Selection of cases for reporting takes place in individual hospitals within the total seen and this must be considered as well as the usual selection which takes place by method of reference of patients to those hospitals in the first place. Arbitrary decisions must be taken about classification in doubtful cases ; it is important, therefore, to give a picture of the adjacent tumours from which a selection has been made if valid comparisons are to be attempted In a hospital where the results of treatment of patients with tumours of the laryngo-pharynx (Lederman, 1954) or stomach are also being reported, selection internally is not likely to be biased in favour of including a few extra successfully treated patients from either anatomical extreme. The few survivors are wanted on both sides of the dividing lines and cannot decently be put into two separate site classifications when reporting the work done. In our own case, for example, if we consider the groups shown in Figure 1, there were seven five-year survivors out of the 125 patients with epi-oesophageal tumours (five treated by irradiation, one by irradiation followed by surgery, and one by surgery) and of the ninety-four patients with tumours involving the cardia, one survived for nearly nine years after insertion of radon seeds, and there were two patients who survived for four years after surgery

The classification adopted here has been based for the upper end on that of Lederman (1954), and for the lower end has been my own (Smithers, 1956). Figure 1 shows the internal selection which has operated in the Royal Marsden Hospital to produce the 314 new patients with
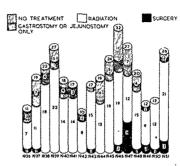
Fig. 2. Carcinoma of the oesophagus—Royal Marsden Hospital, 1936-1951.
Incidence of treatment method used each year.
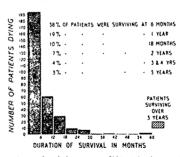
Fig 3 Distribution of survival times among 314 treated and untreated patients with carcinoma of the oesophagus seen at the Royal Marsden Hospital, 1936-1951.

(156)

to do something for all these patients unless their general condition was so poor or their disease so advanced that even an attempt at temporary relief of dysphagia seemed unwarranted. Of the sixty-five patients classified as " untreated " thirty-three had either a Souttar's tube inserted, a gastrostomy or jejunostomy, or some chemotherapy. The fate of the " untreated " patients is shown in Table III, 95 per cent. dying within six months and none surviving two years. Those treated by resection appear in Table IV ; there are only twenty, and 60 per cent. died within six months, 5 per cent. living more than two years. Table V gives those treated by irradiation, the major part of the total, 229 patients : 52 per cent. died within six months and 9 per cent. lived for more than two years.

The trend in choice of treatment over the years is shown in Figure 2, and Figures 3, 4, 5, and Tables VI and VII show the results in different forms.

TABLE 1

SELECTION BY METHOD OF REFERENCE AMONG 314 NEW PATIENTS WITH CARCINOMA OF THE OESOPHAGUS SEEN AT THE ROYAL MARSDEN HOSPITAL, 1936–1951

Means of reaching Joint Consultation Clinic	Hospital Patients	Private Patients
	28 (11%)	13 (22%)
	10 (4%)	9 (15%)
	54 (21%)	4 (6%)
	24 (9%)	1 (2%)
Physician	92 (36%)	7 (12%)
Old Patients previously attending	9 (4%)	2 (3%)
Method of Reference not recorded	37 (15%)	24 (40%)
TOTAL	254	60

TABLE II

NEW PATIENTS WITH CARCINOMA OF THE OESOPHAGUS SEEN AT THE ROYAL MARSDEN HOSPITAL, 1936–1951

Years	Number seen	Not Treated	Treated	Per cent. Treated
1936–1939	79	19	60	76
1940–1943	62	10	52	84
1944–1947	102	29	73	72
1948–1951	71	7	64	90
TOTAL	314	65	249	79

THE TREATMENT OF CARCINOMA OF THE OESOPHAGUS

TABLE VI
SURVIVAL OF ALL NEW PATIENTS WITH CARCINOMA OF THE OESOPHAGUS SEEN AT THE ROYAL MARSDEN HOSPITAL, 1936-1951

Year	Number of Patients seen	1 yr.	2 yrs	3 yrs.	4 yrs.	5 yrs.	6 yrs	7 yrs.	8 yrs	9 yrs.	10 yrs
1936	11	1	1	1	1	1	1	.1			
1937	19	4	1								
1938	22	7	3	2	2	2	1	1	1		
1939	27	4									
1940	18	3									
1941	17	3	1								
1942	10	1	1	1	1						
1943	17	3	2	2	1	1					
1944	19	3									
1945	24	2	1								
1946	32	4	1								
1947	27	6	3	2	2	2	2	1	1		
1948	20	4	3	3	3	2	2	2			
1949	12	5	1	1	1	1					
1950	13	2	2	1	1	1					
1951	26	7	1								
TOTALS	314	59	21	13	12	10	6	5	2		
PER CENT.	100	19	7	4	4	3	2	2	1		

TABLE VII
CARCINOMA OF THE OESOPHAGUS
NEW PATIENTS TREATED AT THE ROYAL MARSDEN HOSPITAL, 1936-1951

Treatment	Number of patients treated	Survivors for more than 5 years
X-rays	198	7 { 2 died with cancer present at 7½ and 5½ years. 4 died of intercurrent disease without clinical evidence of cancer at 5½, 6, 7, and 9 years. 1 alive without clinical evidence of cancer at 8½ years.
Radium	31	2 { 1 died with cancer present at 7 years 1 alive without clinical evidence of cancer at 5½ years.
Surgery	20	1 alive without clinical evidence of cancer at 8 years*
TOTAL	249	10

*Operated on by Mr R. W. Raven in 1948.

TABLE III

CARCINOMA OF THE OESOPHAGUS—ROYAL MARSDEN HOSPITAL 1936–1951
TIME OF DEATH OF PATIENTS NOT TREATED

Years	Number not treated	Died within 3 months	Died within 6 months	Died within 1 year	Died within 2 years	Surviving more than 2 years
1936–1939	19	13	4	1	1	0
1940–1943	10	6	4	0	0	0
1944–1947	29	18	10	1	0	0
1948–1951	7	6	1	0	0	0
TOTAL	65	43	19	2	1	0
PER CENT.	100	66	29	3	2	0

TABLE IV

CARCINOMA OF THE OESOPHAGUS—ROYAL MARSDEN HOSPITAL, 1936–1951
TIME OF DEATH OF PATIENTS TREATED BY RESECTION

Years	Number treated	Died within 3 months	Died within 6 months	Died within 1 year	Died within 2 years	Surviving more than 2 years
1936–1939	2	2	0	0	0	0
1940–1943	1	1	0	0	0	0
1944–1947	10	4	1	2	3	0
1948–1951	7	4	0	0	2	1
TOTAL	20	11	1	2	5	1
PER CENT.	100	55	5	10	25	5

TABLE V

CARCINOMA OF THE OESOPHAGUS—ROYAL MARSDEN HOSPITAL, 1936–1951
TIME OF DEATH OF PATIENTS TREATED BY IRRADIATION

Years	Number treated	Died within 3 months	Died within 6 months	Died within 1 year	Died within 2 years	Surviving more than 2 years
1936–1939	58	17	15	11	10	5
1940–1943	51	9	21	11	6	4
1944–1947	63	13	20	18	7	5
1948–1951	57	16	9	17	9	6
TOTAL	229	55	65	57	32	20
PER CENT.	100	24	28	25	14	9

Comparison of treatment results

Unfortunately I can find no account of any group of patients treated for cancer of the oesophagus in which there is any description of either the external or internal methods of selection of the cases reported. The results chosen to be shown here are divided into two classes : (1) those including all patients in whom a diagnosis of carcinoma of the oesophagus was made, whether treated or not—this offers some reasonable basis for comparison if site incidence is taken into account ; and (2) those reporting only the treated patients—these are of interest but not really suitable for comparisons. All other papers reporting results in specially selected personal or treatment groups have been omitted. Table VIII shows the results obtained in hospitals recording all patients seen, where a comparison may be attempted, and Table IX those in hospitals only recording all the patients treated. In the table including all patients seen, the proportions treated by surgical resection and by radiotherapy are shown together with the treatment method employed for each of the five-year survivors.

At first sight it might appear that rather similar results are now being obtained by both surgery and radiotherapy in those centres where careful recording is practised. The differences, however, merit closer examination. In these reports, although we are unable to compare the methods of selection from tumours of adjacent sites, we can examine the differences in site distribution within the reported groups for indications of the sort of selection which may have occurred. When we do this it is at once clear that lower third tumours predominate in the surgical series, while those in the radiotherapy series are much more evenly sited and contain a slightly higher proportion of upper third tumours. A few groups have been chosen to illustrate this point (Fig. 6).

When we examine the individual results we see that surgery gets its best results in the lower third and radiotherapy in the upper third. There is, therefore, bias in selection within a hospital for these sites according to the treatment method predominating there, as well as selection from without by reference of patients suitable for the particular form of treatment known to be in favour or the particular skills known to be available. If we look at Sweet's surgical results (Table X), for example, we see that his five-year survival rate for carcinoma of the lower oesophagus and cardia is 15 per cent. but for the cervical oesophagus is nil ; at the Royal Marsden Hospital where radiotherapy predominates at this site, almost the reverse situation exists (Table XI). Knowing that series such as these two represent very different selection from the total of persons suffering from this disease, we must be extremely careful in drawing any general conclusions about the treatment of tumours at all sites in the oesophagus from the overall figures produced by either surgery or radio-therapy alone.
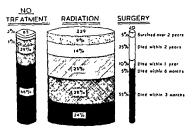
Fig. 4. Carcinoma of the oesophagus—Royal Marsden Hospital, 1936-1951.
Survival of patients according to method of treatment used.
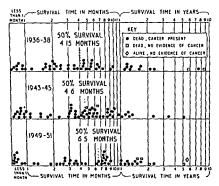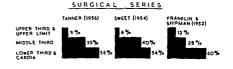
Fig. 5. Carcinoma of the oesophagus—Results obtained at the Royal Marsden
Hospital for the three periods, 1936-1938, 1943-1945, and 1949-1951.
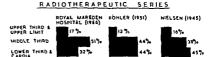
Fig. 6   Carcinoma of the oesophagus.   Proportionate distribution by site in selected surgical and radiotherapeutic series.

If we take all the reported five-year survivors following any treatment for cancer of the oesophagus which we can find, irrespective of whether they come from hospitals reporting all patients seen or not, and chart these by site and treatment method (Fig. 7) we see at once how this pattern is maintained.   We do not know how many of the lower third tumours treated by surgery were adenocarcinomas and might have been classified by others as arising in the stomach.   Surgery, nevertheless, is gaining its

SITE	TREATMENT	
	SURGERY	RADIOTHERAPY
UPPER THIRD	2	33
MIDDLE THIRD	7	15
LOWER THIRD	65	13
TOTAL OF 5 - YEAR SURVIVORS	74 (COMBINED TREATMENT CASES OMITTED) 61	
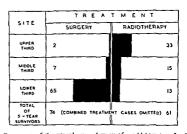
Fig. 7   Carcinoma of the oesophagus   Survey of world literature for five-year survivors classified by site and treatment.

The 135 cases included in this Figure are those reported in papers starred in the list of references

TABLE VIII

RESULTS OF TREATMENT OF CARCINOMA OF THE OESOPHAGUS IN CENTRES REPORTING ALL PATIENTS SEEN

Hospital and City	Years	Total No. of Patients seen	Percentage untreated	Percentage resected	Percentage irradiated	Number surviving 2 years	Number surviving 5 years	Treatment of survivors	Author
Memorial Hospital, New York	1931-1955	1233	24.2	11.1	64.7	34	12	R 6 / S 3 / R 1 S 3	Watson & Goodner (Personal Communication, 1956)
Royal Marsden Hospital, London	1936-1951	314	20.7	6.4	72.3	21	10	S 1 / R 9	Present Report
United Hospitals, Birmingham	1936-1951	703	42.1	17.4	40.5	13	2	S 2	Vol. I, Ann Cancer Rpt 1953 & Pers Comm, 1956
Mount Sinai Hospital, New York	1936-1952	457	57.1	42.9	0.0	27	11	S 11	Garlock & Klein, 1954
Veterans Admin Hospital, Hines	1939-1954	489	37.0	13.3	49.7	6	—		Purstow, Gillesby & Guynn, 1955
Chicago (Hospital) & Holt Radium Inst, Manchester	1940-1944	260	45.4	0.0	54.6	—	—		Third Stat. Report, Holt Radium Inst, 1950
Radiumhemmet, Stockholm	1940-1950	691	40.8	0.3	58.9	18	10	R 10	Hultberg (Personal Communication), 1956
Swedish Hospital, Seattle	1940-1950	59	47.5	0.0	52.5	—	3	R 3	Buschke (Personal Communication), 1956
Yale University School of Medicine, New Haven	1940-1950	180	60.0	16.7	23.3	—	3	S 3	Shedd, Crowley & Lindskog, 1955
Roper Hosp, Charleston	1940-1951	170	78.2	17.7	4.1	1	—		Parker, Hanna & Postlethwait, 1952
St. James' Hosp, London	1944-1951	160	58.1	41.9	0.0	16	6	S 6	Tanner (Personal Communication), 1956
Radiotherapy Dept, University Hospital, Lund *	1944-1952	249	13.3	0.0	86.7	28	12	R 12	Eberius & Cronqvist, 1946
Lahey Clinic, Boston	1945-1949	161	57.1	42.9	0.0	20	3	S 3	Adams, Hare, Davis, Trump & Grant, 1953
Chiba University School of Medicine, Chiba	1946-1953	1202	—	31.1	—	94	18	S 18	Nakayama (Personal Communication), 1956

R —Radiotherapy, S =Surgery (Resection)

NOTE.—Authors differ about including tumours of the cardia among those in the lower oesophagus, and in several groups every survivor has not yet been followed for five years. Tumours at all levels of the oesophagus are included in the other series in this table.

* The tumours reported in this series of patients are defined as being intrathoracic and presumably none was situated in the cervical oesophagus.

successes predominantly in the lower third of the oesophagus where access and reconstruction are easiest, and radiotherapy in the upper where involvement of inaccessible lymph nodes below the diaphragm is least common. While so little success is obtained with tumours in the middle of the oesophagus and while radiotherapy continues to get rather more survivors than surgery in this group, its other advantages weigh heavily in its favour as the method of choice for these patients. With the minimum of discomfort, with a stay in hospital of a few weeks (usually most helpful for a starving patient who can then be properly fed and cared for) and with virtually no treatment mortality, the majority of these patients, treated by modern methods, swallow well again until their death. Their total survival time in comfort greatly exceeds that currently secured for the surgical group.

The over-simplified statements that either surgery or radiotherapy because it produces a 15 per cent. five-year survival rate is therefore the treatment method of choice for patients with oesophageal cancer at whatever site it arises, are grossly misleading. The depressing statement that what we are seeing now with our few five-year survivors from either treatment method is merely the long term end of the natural history of the disease is nonsense unless this natural history is changing, since these survivors were not to be found before the introduction of modern surgical and radiotherapeutic methods. The more serious suggestion that most of the survivors following radiotherapy really had tumours of the laryngo-pharynx and most of those following surgery tumours of the stomach, is at first perhaps a little more difficult to refute. These patients, however, have been successfully treated for cancer whatever our opinion may be about detailed anatomical classification of site of origin, and a close look at these reports leaves little doubt that the majority now so reported are indeed primary oesophageal tumours.

For proper comparison of results far more attention needs to be paid to selection both within and without the reporting hospitals. For improvement in results real co-operative work between surgeons and radiotherapists is required to keep the balance. Both methods are likely to improve still further in the next few years, they have their individual sites of maximum advantage, but competition to the patient's disadvantage instead of co-operation to produce the best results is still far too common. There would seem to be a place for combined treatment in some cases either by pre-operative irradiation or by the use of radioactive gold grain implants (Fleming and Barrett, 1955). Since, without some radical change in getting the more favourable patients earlier to hospitals for their treatment, no great further advance can be expected with either method, a careful selection is required by surgeons and radiotherapists together of the best possible treatment for each patient on existing evidence, rather than on future hope, unwarranted optimism, or unshakable faith in personal skill

TABLE IX

RESULTS OF TREATMENT OF CARCINOMA OF THE OESOPHAGUS IN CENTRES REPORTING
ALL PATIENTS TREATED

Hospital and City	Years	Total No of patients treated	Number surviving 2 years	Number surviving 5 years	Treatment of survivors	Author
University Radiotherapy Clinic, Zürich	1919-1951	R.724	> 18	7	R 7	Schärer, 1955.
University of Wisconsin Medical School, Madison	1928-1941	R 85	3	1	R.1	Pohle and Benson, 1943.
Christie Hospital & Holt Radium Institute, Manchester	1934-1938	R 90	—	2	R.2	Second Stat Report, Holt Radium Inst, 1945.
Central Institute of Radiotherapy, Helsinki	1936-1949	R 296	8	2	R 2	Kohler, 1951
Massachusetts General & New England Deaconess Hosps., Boston	1939-1952	S 303	59	18	S 18	Sweet, 1954
Radium Centre for Jutland, Aarhus	1941-1947	R 217	12	1	R 1	Krebs, Nielsen and Andersen, 1949

R. = Radiotherapy ; S. = Surgery (Resection)

NOTE Authors differ about including tumours of the cardia among those in the lower oesophagus, and in several groups every survivor has not yet been followed for five years.

TABLE X

CARCINOMA OF THE OESOPHAGUS—SWEET, 1939-1947
FIVE-YEAR SURVIVAL BY SITE

Site	Number operated on	Survived five years	Per cent.
Cervical-Oesophagus	Less than 8	0	—
Mid-Thoracic Oesophagus	70	2	3
Lower Oesophagus and Cardia	109	16	15

TABLE XI

CARCINOMA OF THE OESOPHAGUS—ROYAL MARSDEN HOSPITAL, 1936-1951
FIVE-YEAR SURVIVAL BY SITE

Site	Number seen	Survived five years	Per cent.
Upper Third, including Upper Limit	52	6	11·5
Middle Third	159	2	1
Lower Third, including Lower Limit	102	2	2

NOTE.—In two patients the site was not recorded. One patient had two tumours (upper limit and middle third).

BLADDER TUMOURS

by

D. M. WALLACE

A REVIEW of the available statistics relating to tumours at various sites in the body will naturally give the impression that cancer at all sites is increasing, partly due to more accurate diagnosis and partly due to there being a greater number of persons at risk in each age group.

In order to obviate these possible errors Case (1956) has plotted the age of death of persons born in different decades from different forms of cancer. Cancer of the lung is thus shown to be causing death in greater numbers in progressively younger male age groups. Tumours of the bladder, i.e. carcinoma and papilloma combined, are also behaving in a very similar fashion. For each decade of men at risk deaths are occurring from this disease at an increased rate in the younger age groups.

Clemmensen (1956), working with the morbidity returns of the Danish Tumour Registry, has been able to show a similar increase, but the Danish figures which are cases recorded while still alive, not death certification, suggest that the increase is occurring mainly in one group, the males living in towns.

This selective increase in one group of the population raises the possibility of there being a common extraneous factor, such as environmental exposure as would be encountered in industry or a factor of changing social habits, especially diets, smoking, drinking, etc.

The value of an accurate history, both of the patient's occupation and of his habits, cannot be exaggerated, since only by examining adequate histories of large numbers of cases retrospectively will it be possible to select possible causative factors.

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D. W. SMITHERS

ACKNOWLEDGMENT

REFERENCES

* The papers starred in this list refer to the 135 cases included in Figure 7

ADAMS, H. D., HARE, H. F., DAVIS, W. L., TRUMP, J. G., and GRANKE, R. C (1953) *Ann Surg* 138, 631.

142, 2

t *8th Int. Radiol. Cong*, Mexico

p 306.

LIEDERMAN, M.

55

1916-49, pp. 14-21   * *Report* 1, Alimentary Tract.

(1956) Personal communication from the Follow-up Department.

*WATSON, W. L., and GOODNER, J. T (1956) Personal communication.

INVESTIGATION OF BLADDER TUMOURS

① <u>Culture of urine</u> — A sterile urine at the first examination
eliminates the factor of sepsis

② <u>IV Pyelogram</u>

This may show — filling defects
renal function
stasis of ureter

③ <u>Cystoscopy under anaesthetic</u>

For Type ~ papillary or solid
Size ~ small average or large
Base ~ pedunculated or sessile
Surrounding mucosa

④ <u>Biopsy</u>

Resectoscope   Cold Punch   Lowsley's or Riches' forceps

⑤ <u>Bimanual under anaesthetic</u>

Complete relaxation and empty bladder

Palpable but   Palpable but   Palpable but   Palpable but
mobile inside   infiltrating   extravesical spread   fixed to pelvis
bladder   though still mobile
                                             in pelvis

Fig. 38   Clinical Assessment of Bladder Tumours. There are five
main sections for a complete assessment. Omission of any one of
these may mean inaccurate diagnosis. (From *Ann. Roy. Col.
Surg. Eng*)

Classification of Tumours

During the last decade the treatment of bladder tumours has tended to swing from radical surgery to either conservative surgery or to radiotherapy. Although this change in the approach to the problem has been partially due to the elaboration of new techniques in the field of radiotherapy, it is also partly due to the better understanding of the disease process and to the appreciation of the natural history of the disease.

Confusion has been caused by the numerous different methods of presenting results. Some series have been classified according to the histological type of tumour without reference to the extent of spread. Other series have been classified according to the extent of spread without reference to the histological type. Few series have included both factors.

Assessment of the spread may be a clinical one or it may be a pathological one. It is obvious, however, that a pathological staging, although more exact, is only applicable to cases where the tumour has been removed and, therefore, results based on this method cannot be compared with results of conservative therapy, whether endoscopic, radiotherapeutic or chemical.

It is only by applying a clinical assessment to all tumours and staging each case on the result of the clinical examination as honestly as possible that comparisons can be effected between various methods of treatment.

The pathological staging, that is the extent of spread of a tumour as determined by the examination of an operative specimen, is an essential check on the accuracy of the clinical stage, but as it can only be applied to cases treated surgically it cannot be used for all series. The pathological staging will be more accurate for prognosis, but even this will vary with the extensiveness of the operation—one case treated by partial cystectomy only would be less accurately assessed than a similar case treated by total cystectomy and radical excision of regional nodes.

The clinical staging of any bladder tumour will depend on the evidence collected as the result of a clinical assessment (Fig. 38).

site for taking the biopsy better with an instrument he knows rather than one with which he is not fully acquainted. Punch forceps may produce better specimens, but in the presence of bleeding it may be difficult to identify the best portion of the tumour for a biopsy with any of these instruments. Lowsley's forceps, used in conjunction with a resectoscope sheath, is probably the best instrument for tumours of the posterior wall while Riches' forceps is better for the trigone, lateral or anterior walls.

The second factor which affects the grading is the number of groups which are used. Any classification having a large number of grades will have a lower correlation rate than a simpler classification with fewer grades.

The third factor influencing grading is the personal variability of the pathologists so that, if possible, grading of biopsy material and grading of tumour material should always be undertaken by the same pathologist.

The Institute of Urology has suggested the following histological grades.

Papilloma. Where the fronds are delicate, the cell layer 3–4 cells thick, the nuclei pale staining and regular, the cell outlines well defined and of approximately the same size, and where the basement membrane is intact (Fig. 39).

Papillary Carcinoma Differentiated. Where there is more disorder in cellular arrangement, where the cell layer is thicker and the regular palisade arrangement has been distorted, where the cells vary in size, the nuclei stain irregularly and mitoses may be seen. Although it is not seen in every case, a breakthrough of the basement membrane of the papillary process is indicative of a papillary carcinoma rather than a papilloma (Fig 40).

Papillary Carcinoma Anaplastic. Where there is gross irregularity of the cell layer, nuclei vary in shape, size and staining characteristics, mitoses are common, the basement membrane is broken through or cannot be clearly distinguished and the papillary processes themselves are misshapen and distorted (Fig. 41).

Solid Transitional Cell Carcinoma Differentiated. Where the papillary differentiation has been lost but where there is a reasonably well-defined growing edge to the tumour, where invasiveness is not

Clinical Assessment

This method of grouping comparable cases together depends upon a clinical assessment of the spread of the tumour, and upon a study of an adequate amount of tumour tissue removed for biopsy examination. The clinical assessment depends on five main examinations.

1. *Culture of the Urine.* A sterile cystitis may be the early symptom of mucosal carcinomatosis.

2. *Intravenous Pyelogram.* This may reveal abnormal kidney function, dilatation of the ureter, or a filling defect in bladder or ureter.

3. *Cystoscopy.* The main features are size, surface (papillary, solid or ulcerative), single or multiple lesions, pedunculated or sessile and the presence of abnormal mucosa.

4. *Biopsy Examination.* Taken either by a resectoscope or by one of the punch instruments—Riches', Lowsley's, or the Thompson prostate punch.

5. *Bimanual Examination under Full Surgical Anæsthesia.* A few of the papillary tumours, especially the small ones, may be impalpable, but even the papillary tumours with early infiltration can usually be felt if sufficient care is taken during the examination. The bimanual findings may be classified as impalpable, palpable but freely mobile within the bladder, induration of the bladder wall but no evidence of tumour in perivesical fat, a nodular mass in the perivesical fat but still freely mobile, or a mass that is fixed to the pelvic wall. Jewett (1946) was the first to emphasise the value of a routine bimanual examination under full surgical anæsthesia and he was also the first to draw attention to the importance of infiltration as the main factor in prognosis.

Histological Grading of Biopsy or Operative Material

The value of grading from biopsy material has been doubted by several authors. There are, however, several factors which may result in inaccuracy.

Firstly, the method. A diathermy loop may result in charring of the material, but an experienced resectionist will be able to pick the

Fig. 41. Papillary carcinoma anaplastic.

Fig. 42. Solid carcinoma differentiated.

FIG. 39. Papilloma.

FIG. 40. Papillary carcinoma differentiated.

Adenocarcinoma. A relatively rare group—incidence reports vary between 0·4 per cent to 2 per cent of all cases. These may arise from the urachal remnant or as a result of glandular metaplasia. Adenocarcinoma is, however, more frequently due to metastatic deposits from an extravesical primary tumour, such as stomach, rectum, colon or prostate.

Clinical Stage

The clinical stage of a tumour depends on an assessment of all available evidence. If certain parts of the examination have been overlooked it may be that the clinical stage recorded will be underestimated.

The Union Internationale contre le Cancre has suggested that the principles of clinical staging should be based on

> T = evidence of direct spread of the *tumour,*
> N = evidence of invasion of regional *nodes,*
> M = evidence of distant *metastasis.*

The bladder is not, however, an organ whose regional nodes can be readily palpated and, therefore, the suggested staging is based purely on the evidence of direct spread, although when other information may be available it can be added under the appropriate section (Denoix, 1954).

Evidence of Direct Spread

Mucosal Stage (*T*1). The tumour is cystoscopically apparently confined to mucosa. A mass, if palpable, is freely mobile inside the bladder. The biopsy examination shows no evidence of infiltration and the intravenous pyelogram shows no dilatation of the ureter, although slight stasis or delay is often associated with mucosal lesions (Fig. 44).

Muscular Stage (*T*2). Cystoscopically there may be cicatricial bands; on bimanual examination there is usually a diffuse, rubbery thickening involving a portion only of the bladder wall; biopsy material may show evidence of muscular infiltration, and the pyelogram, should the tumour be situated near the ureteric orifice, will show moderate or gross dilatation of the ureter if the intramural portion is involved in growth (Fig 45).

prominent, where the cells and nuclei, arranged in a fairly orderly fashion, are of approximately the same size, shape and staining characteristics (Fig. 42).

Solid Transitional Cell Carcinoma Anaplastic. Where there is gross cellular irregularity, prominent invasive characteristics, with no well-defined margin, and where the nuclei vary in size, shape and in the intensity of staining (Fig. 43).
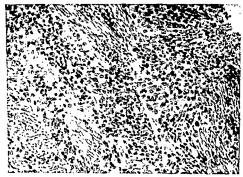
FIG. 43. Solid carcinoma anaplastic.

Squamous Cell Carcinoma. There are cell nests, keratinisation and prickle cells predominate. The general picture, however, must be homogeneous throughout since a solid anaplastic tumour with metaplasia may mimic this histological picture. It is important to distinguish between these two types of tumour, since the solid anaplastic disseminates rapidly, while the squamous-cell carcinoma disseminates late and usually kills by local interference with kidney function.

Other classifications that have been used·are:
Jewett (1946)

    Stage A = mucosal
        B_1 = superficial ⎫
        B_2 = deep   ⎬ muscular
        C = perivesical.

Marshall (1956) (Fig. 48)

        O = ⎫
        A = ⎬ mucosal
        B_1 = ⎱ superficial ⎫
        B_2 = ⎰ deep   ⎬ muscular
        C = perivesical

        D_1, D_2 = any stage with nodal involvement, local or distant.

Dukes, Masina (1949)

        1a = mucosal
        1b = muscular
        2 = perivesical
        3 = perivesical with glands
        4 = pelvic fixation or any stage with metastases or more
            than regional node involvement.

Riches (1956)

        A = mucosal
        B = muscular and perivesical
        C = perivesical with nodes or pelvic fixation.

Poole-Wilson (1954)

    Superficial = mucosal and muscular
    Advanced = perivesical

The need of a uniform classification is paramount to enable comparison between series of cases. The T.N.M. International would appear to fulfil this need.

Bladder Tumours as an Industrial Hazard

As there has been a considerable increase in the incidence of bladder tumours in the younger age groups the question of industrial or environmental factors must be considered in all cases of tumour. It has now been realised that the " aniline dye-stuff workers " are not unique in the liability to contract bladder tumours, and that the

Perivesical Stage (T3). There is a discrepancy between the size of tumour seen cystoscopically and the size felt bimanually. There is more tumour outside the bladder than inside. The external surface may be nodular and the mass is hard but completely mobile in all directions in the pelvis (Fig. 46).

FIG. 44. Mucosal stage.

FIG. 45. Muscular stage.

FIG. 46. Perivesical stage.

FIG. 47. Pelvic fixation or involvement of adjoining organs.

Pelvic Fixation (T4). The involvement of scar or adjoining organs, or tethering of the growth to the anterior or lateral walls of pelvis usually means that the growth is irremovable, without hope of cure, although technically removable for palliative reasons only (Fig. 47).*

* The T.N.M. staging can be amplified, even at a later date, by the addition of information about nodes, Na impalpable, Nb palpable or N + or — histologically positive or negative, or by additional information about liver, lung or bony metastases = M.

xenylamine are both readily obtainable and are being used in a considerable variety of industrial products.

Melick (1954) in a recent paper has reported an incidence of 11 per cent of bladder tumours in workmen exposed to xenylamine, a chemical which was forecast as a possible carcinogen by Case (1954), and Walpole, Williams and Roberts (1952). Benzidine, also a constituent of dye processes, is a well-recognised carcinogenic risk, so much so that bottles of this chemical are prominently labelled as carcinogenic by the manufacturers.

TREATMENT OF BLADDER TUMOURS

By far the greater number of bladder tumours can be treated by perurethral means, either by endoscopic resection using the loop or the punch, or by coagulation. The point at which various surgeons will select other methods will depend on multiplicity of tumours and infiltration. The presence of infiltration into the muscle layers is usually taken as a contra-indication to further endoscopic surgery. A bladder mucosa which is grossly abnormal, the site of multiple tumours both in space, i.e. occurring simultaneously, or in time, i.e. occurring at different sites over a period of time, may be considered as unsuitable for further endoscopic therapy.

Milner (1953) and Chapman (1954) have both produced series of cases showing what can be achieved by endoscopic methods.

Multiple mucosal tumours with areas of abnormal mucosa, often incorrectly termed papillomatosis, are usually considered to be beyond adequate control by diathermy. These cases may be treated by either total cystectomy or by some form of irradiation. Total bladder irradiation by external therapy has been advocated, but satisfactory statistics are not yet available. It is, however, well recognised that external irradiation can effect a complete disappearance of these tumours in an appreciable percentage of cases.

Intracavitary Irradiation

The insertion into the bladder of radioactive material has been tried now for some years. These sources may consist of solid radium

risk is not due to the aniline as such but to the use of intermediate chemicals in the dye processing. It is these compounds, mostly aromatic, amino and basic in form (Goldblatt, 1947), that are responsible for the production of bladder tumours, since other industries are also finding bladder tumours in the workers exposed to these chemicals. The full occupational history of any patient
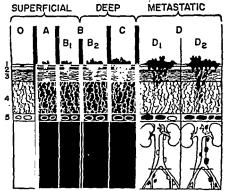
Fig. 48. Marshall's clinicopathological classification. This staging can only be fully applied to cases treated by radical surgery when both nodes and bladder are available for pathological study. (Copied from *Cancer*.)

with bladder tumours should be taken as a routine procedure, for although a sudden strong exposure to a chemical may produce symptoms of cystitis with hæmaturia very rapidly, there is usually a long latent period between the first exposure and the development of a tumour. In the case of β-naphthylamine it is usually many years.

Although the production of β-naphthylamine has been stopped in Britain it is still being produced abroad, while benzidine and

Instilling a radioactive colloidal solution into the bladder without a balloon has considerable theoretical advantages, since a fronded lesion will project into the solution and the fronds will, therefore, be irradiated on both sides. If a colloidal solution is used the risk of absorption is minimal, and if the radioactive material (gold, yttrium) is mainly a beta emitter only, a very superficial treatment will be given. The conservatism of the muscle of the bladder wall, and the
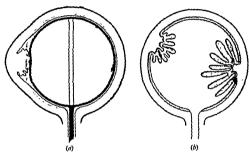
FIG. 49. Intracavitary irradiation with isotope solutions.
(a) *Balloon:* base of a bulky tumour is displaced out of zone of effective irradiation.
(b) *Colloidal solution.* Solution permeates between fronds but leaves small volume at base of a sessile tumour unirradiated.

prevention of changes in the arterioles of the submucosal layers are both important if post-therapy morbidity is to be avoided.

Interstitial Irradiation

The treatment of single tumours of the bladder which have begun to infiltrate but have not yet spread through the bladder wall (T2) can be treated either by partial cystectomy or by interstitial irradiation.

Interstitial irradiation can be divided broadly into two main

by traction. The bulkiness of a nylon sheath, which is required for the elimination of β-radiation, renders the cobalt sutures more cumbersome than the tantalum wire where the filtration is effected by platinum (Vermooten, 1955).
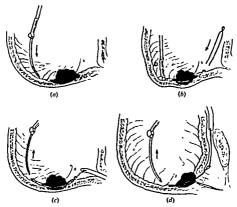
Fig. 51. Insertion of tantalum wire.

(a) Needle inserted into bladder wall clear of the edge of tumour.
(b) Needle in position in bladder wall—hairpin of tantalum being inserted.
(c) Needle withdrawn—tantalum lying in bladder wall and loop tied to urethral catheter.
(d) Wire inserted for tumour on anterior wall or bladder neck, via retropubic space—withdrawn with retropubic drain.

One of the criticisms levelled against interstitial implants is that frequently the tumour is completely removed by diathermy loop excision prior to implantation and, therefore, the result may be due to the excision and not to the implants. Several cases have been

groups: the removable implant such as radium, tantalum (Fig. 50) or cobalt wire; and the irremovable implant, such as radon, gold grains or chronic phosphate solution.

Radium is readily available and can be used even in the smallest centres, but whether the results obtained warrant universal use is debatable (Higham, 1955; Darget, 1951).

FIG. 50. X-ray of tantalum wire inserted into base of tumour.

Tantalum is a flexible wire coated with platinum to filter off the β-radiation. It may be regarded as " flexible radium " since it can be introduced by means of a twin boomerang needle into the base of a tumour and subsequently withdrawn down the urethra (Fig. 51). With a long half-life (four months) it can be always available even at short notice (Wallace, Stapleton, and Turner, 1952).

Cobalt wire sheathed in nylon tubes is again flexible and can be darned through the base of a tumour, being subsequently removed

Partial Cystectomy

Partial cystectomy, or, as it is also sometimes called, segmental resection, is viewed with disfavour in certain clinics, largely due to the disappointing results that have been reported over recent years. Where, however, a correct selection has been made, and where care has been used to operate only on cases that conform to the criteria detailed below, the results are comparable, if not better, than the results of cases treated by more radical methods (Masina, 1953).

The ideal case for partial cystectomy must be a single lesion showing no evidence of abnormal mucosa, patches of hyperplasia or a second " seeding " lesion. The margins of the growth should be well defined, without any suggestion of submucosal spread. The position of the lesion should be such that a clear margin of at least an inch can be obtained between the line of section and the histological edge of the tumour.

The main contra-indications to partial cystectomy are multiple tumours, or evidence of mucosal instability throughout the bladder, or a tumour with an edge so ill defined that it is impossible to say where tumour ends and normal bladder wall begins.

The multifocal tumours are evidence of a mucosal disease and any method that fails to treat the entire disease field is doomed to failure. A tumour with an indefinite margin is frequently of a relatively anaplastic type, tending to spread by veins or lymphatics under the mucosa for a considerable distance. Any form of conservative surgery in these cases may be inadequate, since the line of section may transgress the tumour substance. In general it may be added that tumours on or near the base of the bladder are unsuitable for partial cystectomy, since an adequate excision in this region, which may necessitate re-implantation of one or both ureters or reconstruction of the internal meatus, is rarely achieved.

Operative Detail

Partial cystectomy is an operation that must be planned well in advance. At the cystoscopy under anæsthesia the probable lines of excision should be planned, especially in relation to the ureteric orifices and the internal meatus. Unless an adequate margin can be anticipated all hope of conservative surgery should be abandoned.

treated by tantalum wire alone without excision of the tumour and complete tumour regression has occurred.

The irremovable implants—radon seeds or gold grains—have one common disadvantage in that when a technically bad implant is made and the radioactive sources are placed too far apart an area of bladder wall will be under-irradiated, and a local recurrence will be inevitable if active tumour tissue was present at this point.

This lack of control of the amount of irradiation is not present in the removable implants, which can be left *in situ* for a longer time if necessary. Both radon seeds and gold grains generally remain *in situ* once they have been inserted, unlike colloidal gold or chromic phosphate which, when injected into a tumour, may remain there or may diffuse into the surrounding tissues. The infiltration technique cannot be checked for radiation distribution by post-operative X-rays as the solutions used are not radio-opaque. Extremely good results may be obtained in the hands of persons experienced in the technique of implantation provided the selection of cases is strict, and tumours which have spread through the bladder wall are not treated by these methods (Millen, 1950).

External Therapy

The role of external therapy is still not clearly defined. Super-voltage plants of two million volts or more can undoubtedly deliver a very large dose to a volume deep in the pelvis and would, therefore, be suitable for perivesical tumours that were unsuitable for other treatment. This method of treatment causes minimal skin reaction or untoward side effects provided the dose is given over a sufficiently long period. Unfortunately it is these perivesical tumours that are most likely to have spread to lymphatic glands and, although super-voltage therapy may control the primary tumour, the outcome of the disease may be decided by the metastases.

Supervoltage therapy can be used for irradiating the pelvic walls in cases where the primary tumour has been removed by local surgery. It may well be that this combination of surgery followed by supervoltage therapy will prove to be the most efficacious combination.

Others prefer to employ conservative methods first and cystectomy only when other methods have failed. It is, however, obvious that only when the limitations of the various conservative methods are appreciated, and only when the natural histories of the more malignant types of the disease are understood, will total cystectomy be placed on a proper footing.

In brief, however, the following are taken for the main indications for cystectomy (Riches, 1956).

(1) Large, bulky, non-infiltrating tumours beyond the scope of cystoscopic diathermy, too bulky for intracavitary irradiation, too extensive for interstitial irradiation and where, owing to the widespread nature of the disease, open diathermy runs the risk of wound implantation.

(2) Infiltrating tumours of the bladder base where the stage is more than muscular, or where the prostate or posterior urethra are involved, especially if there is evidence of multifocal origin such as areas of hypertrophic mucosa or cystitis cystica or glandularis. Infiltrating tumours of the vault or even muscular tumours occurring with prostatic obstruction are also best treated by radical excision. Involvement of the bladder by growths of adjacent uterus, colon or rectum, cannot be treated in any other way than by total cystectomy.

Total cystectomy may be a life-saving measure in cases of really severe hæmaturia where, in spite of all other methods, the bleeding is uncontrolled. It is infinitely better than opening the bladder blindly in the hope of applying diathermy to a lesion which is usually infiltrating. Radio-necrosis or the bleeding that sometimes occurs after external X-ray therapy, where there are changes in the arterioles or atrophy of the mucosa, may necessitate cystectomy even when the bladder is tumour free. Cystectomy is also indicated when a tumour has been treated by local interstitial irradiation, and subsequently a second tumour develops in another portion of the bladder, sometimes of a completely different histological type.

Finally, when a palliative transplantation of the ureters, even if supplemented by external high-voltage therapy, has failed to relieve symptoms, a purely palliative removal of the bladder may be justified.

At the time of the cystoscopy, too, especial care should be directed to the mucosa at the base of the tumour, and also in the remainder of the bladder since it is only if the lesion is strictly localised that conservative surgery can be justified.

Partial cystectomy may be commenced with a bladder distended or empty. The distended bladder may make dissection easier but carries a greater risk of spillage of tumour cells. A preliminary laparotomy should be undertaken in every case with inspection or palpation of the liver, para-aortic nodes, peritoneal surface of the bladder and pelvic nodes. It is only after confirmation of the operability that the bladder should be opened. It should never be necessary to open the bladder in order to assess operability or even to establish the diagnosis.

Total Cystectomy

Total cystectomy, that is removal of bladder, prostate, seminal vesicles and all the paravesical tissues, but not including a block dissection of the iliac nodes, has certain well defined indications which are accepted in the majority of clinics. It must be remembered, however, that this operation carries not merely a high operative risk but also a high morbidity rate in the post operative period. A mortality rate of 10–20 per cent must be expected if this operation is to be offered to all those that might benefit; a lower mortality rate may be an indication of too stringent selection, while a higher mortality rate is indicative of attempts to achieve the impossible.

The operation of total cystectomy is inevitably severe and should only be offered to the elderly when there is no other method of relieving symptoms. In the younger age groups the almost certain loss of potency may be an argument against too facile recommendation of the operation.

Indications

The indications for total cystectomy vary among different clinics. Some authors believe that only by performing radical extirpation of the disease at the earliest opportunity will the results be improved.

the thumb in front pressure is applied to the apex and the urethra cut. This manœuvre is more certain to prevent accidental spillage from the urethra than any clamp yet devised.

It is rarely possible, if an adequate excision has been achieved, to
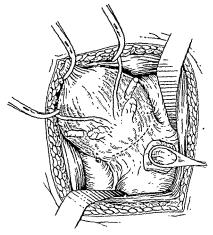
FIG. 52. Exposure of bladder base at total cystectomy showing vasa, ureters and vesicles. Incision between vesicles opens into space of Proust.

reperitonealise the pelvic floor. In many clinics it is believed that a wide open pelvis is less likely to strangulate a loop of intestine than a tense diaphragm, perforated possibly with multiple small apertures through which a loop of bowel may prolapse.

Total Cystectomy, Operative Technique

The modern technique of total cystectomy is based on the description by Millin and Masina (1949). The pre-operative care of the patient includes full biochemical and radiological studies. The toilet of the colon has been simplified by the use of chemotherapeutic and antibiotic drugs, one of the most popular now being a combination of sulphaguanidine and streptomycin given orally three times a day for three days before operation. A high colonic wash-out may be given daily for several days before operation, but never on the day of operation lest the fluid be incompletely evacuated pre-operatively.

The bladder is emptied at the beginning of the operation and a formal laparotomy carried out with careful palpation of the iliac and para-aortic nodes, the liver and both kidneys. If the operation is to be completed in one stage the ureters are defined, sectioned as low as possible and rubber catheters inserted so that urine will drain away from the operative area. There is no harm likely to follow if, at this stage, the internal iliac arteries are tied on each side as in an abdomino-perineal excision of rectum. This step minimises the bleeding from superior and inferior vesical pedicles. The peritoneum is then incised along the pelvic brim to the site of the ureters and joined as low in the recto-vesical pouch as possible. At this point it is as well to remember that the object of the operation is to remove not merely the bladder but all the perivesical tissue, and that to achieve this the block of tissue removed should be thick enough to prevent identification of the bladder wall. A *simple method of dissecting down to the vesicles consists of following the obliterated hypogastric artery to where it crosses the vas, then to dissect between the vas and the peritoneum to the crossing with the ureter, then to trace the ureter to the seminal vesicles. Between the seminal vesicles there is a layer of fascia which is incised to open into the space of Proust (Fig. 52). This space leads forward to the apex of the prostate. The vesical pedicles are clamped and cut and finally the bladder is tethered only by the two puboprostatic ligaments on either side of the urethra.* When these are cut the apex of the prostate can be drawn upwards with resultant elongation of the urethra. With the index finger behind the apex of the prostate and

Papillary tumours of the bladder may co-exist with similar lesion of the kidney; the kidney lesion may precede the bladder lesion or it may develop after the bladder lesion.

Papillary tumours may be of different histological types, existing together in the bladder at one moment in time.

Papillary tumours have been recorded as apparently undergoing spontaneous remission after transplantation of ureters.

Industrial bladder tumours due to exposure to either β-naphthylamine, benzidine or xenylamine are indistinguishable from those tumours which appear to arise spontaneously.

The urogenous (or water-borne factor) theory is based on the supposition that there might be an irritant—possibly of a chemical nature—in the urine which bathes the entire mucosa and initiates a field change over the whole of the urinary tract. If this were true it would explain the presence of mucosal hyperplasia occurring in many places in the bladder before the appearance of a tumour. These changes, sometimes taking the form of a mossy hyperæmic mucosa, cannot be explained by the seedling theory. By analogy with the known industrial tumours it might be supposed that this factor would have some resemblance to the metabolites of β-naphthylamine or to similar carcinogenic compounds, all of which are basic, amino and aromatic in structure.

Experimentally the evidence for the urogenous theory is considerable, based largely, however, on work with β-naphthylamine. The original observation by Hueper, Wiley, Wolfe (1938) that after transplantation of the ureters, feeding dogs with β-naphthylamine will no longer cause production of bladder tumours, was of fundamental importance. Scott (1953), repeating these experiments, confirmed the findings but, in addition, in one animal which developed a stenosis at the uretero-colic anastomosis with hydro-ureter and hydronephrosis, a papillary tumour developed on the ureteric mucosa at the site of maximum stasis.

MacDonald (1954), by dividing the bladder into two halves each with the same blood, nerve and lymphatic supply but only one half in contact with urine, was able to demonstrate that only the bladder mucosa in contact with the urine was liable to develop tumours. In one of his dogs a fistula developed between the two pouches and in

The ureteric transplantation is customarily left to the last, since the delicate stitching and the exact accurate approximation without tension of the colon to the ureter can easily be disarranged if undertaken too early in the operative plan.

Radical cystectomy—that is, the removal of bladder, prostate and vesicles and also all the lymph nodes of the pelvic walls up to the bifurcation of the iliac arteries is more in keeping with cancer surgery elsewhere, but it is an operation that is practised in relatively few clinics (Marshall, 1956). Whether the removal of all the lymph nodes compensates for an inevitably higher operative mortality must await assessment in years to come.

Possible Ætiological Factors of Bladder Tumours

The ætiological factors and natural history of bladder tumours have been investigated by many centres. The older theories have been based on the " seedling " mechanism, the idea that exfoliated cells from a tumour could implant on intact mucosa. MacDonald (1956) has shown that this can, in fact, occur in an empty defunctioned pouch of bladder and several authors have supported this possibility, but in the majority they have qualified the seedling theory by postulating the presence of abraded or traumatised mucosa on to which the exfoliated cells adhere (Boreham, 1956; Ashworth, 1956). There is no doubt that implantation from a papillary tumour can occur readily in the presence of a traumatised mucosa ; implantation into a wound can equally readily occur, but in both cases the exercise of meticulous care and gentle handling of the tumour, using every aid to prevent scattering cells, can result in wound implantation being reduced to minimal proportions.

The alternative theory to the seedlings mechanism is based on a series of clinical observations.

Papillary bladder tumours may be multiple in space, i.e. multiple tumours present at one moment of time over the whole bladder.

Papillary tumours may be multiple in time, i.e. multiple tumours involving the whole bladder but appearing over a period of years.

Papillary tumours may co-exist with areas of widespread epithelial hyperplasia or mucosal changes.

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REFERENCES

ASHWORTH, A. (1956). *Brit. J. Urol.*, **28**, 3

BONSER, G. M., CLAYSON, D. B., JULL, J. W., and PYRAH, L. M. (1954). *Brit. J. Urol.*, **26**, 49

BOREHAM, P. (1956). *Brit. J. Urol.*, **28**, 163

BOYLAND, E., HARRIS, R. J. C., and HORNING, E. (1954). *Brit. J. Cancer*, **8**, 647

BOYLAND, E., WALLACE, D. M., and WILLIAMS, D. C. (1954). *Biochem. J.*, **56**, xxix

BOYLAND, E., WALLACE, D. H., and WILLIAMS, D. C. (1955). *Brit. J. Cancer*, **9**, 62

BOYLAND, E., and WATSON, G. (1956). *Nature, Lond.*, **177**, 837

BOYLAND, E., and WILLIAMS, D. C. (1955). *Biochem. J.*, **60**, v

CASE, R. A. M. (1954). *Brit. med. J.*, **2**, 50

CASE, R. A. M. (1956). *Brit. J. Prev. Soc. Med*, **10**, 172

CHAPMAN, T. L., and SUTHERLAND, J. W. (1954). *Brit. J. Urol.*, **26**, 364

CLEMMENSEN, J. (1956). *Danish Tumour Registry.*

DARGET, R. (1951). *Tumeurs Malignes de la Vessie.* Masson et Cie.

DENOIX, P. F. (1954). *Monographie de L'Institut National d'Hygiene*, No. 5

DUKES, C. E., and MASINA, F. (1949). *Brit. J. Urol.*, **21**, 273

ELLIS, F., and OLIVER, R. (1955). *Brit. med. J.*, **1**, 136

FREEDMAN, M., and LEWIS, L. G. (1949). *Radiology*, **53**, 342

GOLDBLATT, M. W. (1947). *Brit. med. Bull.*, **4**, 405

HIGHAM, A. R. C. (1955). *Brit. J. Urol.*, **27**, 211

HUEPER, W. C., WILEY, F. H., and WOLFE, H. D. (1938). *J. ind. Hyg. Toxicol.*, **20**, 46

Institute of Urology Pathological Broadsheet, No. 1 (1955)

JEWETT, H. J. (1946). *J. Urol.*, **55**, 366

MACDONALD, D. F., and LUND, R. R. (1954). *J. Urol.*, **71**, 560

MACDONALD, D. F., and THORSON, T. (1956). *J. Urol.*, **75**, 690

MARSHALL, V. F. (1952). *J. Urol.*, **68**, 714

MARSHALL, V. F. (1956). *Cancer*, **9**, 543

MASINA, F. (1952). *Brit. J. Urol.*, **24**, 344

MASINA, F. (1953). *Brit. J. Surg.*, **41**, 494

MELICK, W. F., ESCUE, H. M., NARYKA, J. J., MEZERA, R. A., and WHEELER, E. P. (1955). *J. Urol.*, **74**, 760

MILLEN, J. L. E. (1950). *Brit. J. Urol.*, **22**, 430

MILLIN, T., and MASINA, F. (1949). *Brit. J. Urol*, **21**, 108

MILNER, A. (1953). *J. Urol.*, **69**, 657

POOLE-WILSON, D. S. (1954). *Brit. J. Urol.*, **26**, 326

RICHES, E. W. (1956). *Ann. Roy. Coll. Surg. Eng.*, **18**, 178

SCOTT, W. W., and BOYD, H. L. (1953). *J. Urol.*, **70**, 914

VERMOOTEN, V., and MAXFIELD, J. G. S. (1955). *J. Urol.*, **74**, 767

this animal tumours also appeared in both portions of the bladder.

Bonser (Bonser *et al.*, 1954), by developing a technique for implanting pellets into the bladders of mice, was able to show that β-naphthylamine only rarely produced tumours, but that its unconjugated metabolite, 2-amino-1-naphthol, was highly carcinogenic. This experimental evidence all points to the presence in the urine of a chemical, more simple in form than β-naphthylamine, acting directly on the bladder mucosa.

The similarity between the industrial and spontaneously occurring tumours led to a search for some similar agent in the urine of the spontaneous tumour patients. The experimental evidence of Boyland, Harris and Horning (1954) indicates that the metabolites of tryptophan could apparently produce tumours of the urinary tract, especially if these were excreted in conjunction with other similar substances of a more general carcinogenic nature. The metabolic products of tryptophan, kynurenin, hydroxykynurenin, anthranilic and hydroxyanthranilic acids are all amino, aromatic and basic in form and resemble the 2-amino-1-naphthol chemical structure very closely. These chemicals have all been found in considerably increased amounts in the urine of patients with bladder tumours, especially multiple tumours by Boyland (1956) and Price (1956, personal communication) of Wisconsin. The normal urine contains, if any, only a trace of the substances.

These chemicals have also been shown to be carcinogenic by direct implantation into the bladders of mice, using a simplified form of the Bonser technique (Boyland and Watson, 1956). These chemicals would normally be excreted in a detoxicated form in conjugation with a glucuronide, but it has been shown that urine from bladder tumour cases contain large amounts of the enzyme glucuronidase which, in the presence of stasis or concentration, splits the conjugated compound to release the free acid.

Although the evidence is recent it is accumulating. In at least some types of bladder tumour there may be a factor—probably a free aminophenol compound—which is responsible for the initiation, if not the maintenance, of multiple tumours. This may well be the factor responsible for " recurrences " over subsequent years if it is not suppressed or diluted.

VISCERAL LESIONS IN HERPES ZOSTER

BY

R. WYBURN-MASON, M.A., M.D.

The peripheral nerves and posterior and trigeminal sensory nerve roots contain not only various sizes of myelinated, but also unmyelinated " afferent " fibres. Distally the afferent unmyelinated fibres originate (or end) as free nerve endings not only in the epidermis or dermis, but also between the deeper tissues, including muscles, periosteum, joints, and fascia (Ranson and Davenport, 1931, Feindel, Weddell, and Sinclair, 1948) They also arise (or end) on the blood-vessel walls. Some of them are visceral in origin (Kuntz and Hamilton, 1938) All the evidence suggests that in the somatic tissues these fibres conduct impulses which give rise to diffuse, radiating, compelling burning pain (see Fulton, 1949)

In the posterior nerve roots all the afferent unmyelinated fibres of the peripheral nerves, whether they originate in the skin, deep somatic tissues, blood vessels, or viscera, are grouped together laterally Division of the lateral part of these roots cuts the unmyelinated fibres, and now on stimulation of the peripheral nerve distal to the section no autonomic reflex changes occur as they do in intact animals (Ranson and Davenport, 1931, Clark, Hughes, and Gasser, 1935)

Mode of Production of Lesions of Herpes Zoster

The skin lesions of herpes zoster lie in the area of sensory supply of the posterior and trigeminal nerve roots They manifest themselves firstly as a reddening of the skin followed by oedema and blister formation—that is, the appearance of fluid between the epidermal layers Typical inflammatory changes are found in the skin papillae, in the deeper parts of the corium and in the hair follicles Subcutaneous fat may also contain knots of inflammatory cells Lewis (1927), in his classical researches, showed that irritation of sensory nerves and the posterior nerve roots results in vasodilatation and the release of a vasodilator substance (H-substance) probably histamine, in the skin He further demonstrated that in cases of herpes zoster the same substance was released in the affected tissues and that " herpetic and herpetiform eruptions occur as sequels to lesions of the sensory nerve tracts , not only do they follow irritative lesions of the (root) ganglion, but they are produced also by lesions of those tracts distal to the ganglia themselves " It seems probable that the unmyelinated fibres of the posterior roots with free nerve endings in the skin and other tissues and on the blood vessels are those concerned.

Herpes Zoster and Lesions of Deep Somatic Tissues

When zoster is of gangrenous type the lesions extend deeply and lead to much scarring. Glandular swelling and inflammation in the region of the affected skin lesions is usual and may precede the latter (Bichelonne, 1907, Lebel, 1920; Ramond, 1923), so that they cannot be due to secondary infection of the skin The subcutaneous tissues fat, and muscles may contain painful and swollen areas identical in character with " fibrositis " and " myositis " (see Brain, 1931; Wilson, 1940, Ford, 1946). Synovial effusions occur in the affected areas and the joints may become ankylosed with fibrous tissue. The pericapsular joint tissues

are often swollen and painful, and, when the hand is affected this gives appearances indistinguishable from those of rheumatoid arthritis, so-called " periarthritis chronica rheumatica " (Guillain and Pernet, 1910 ; Guillain and Rouvilze, 1913). The affected limb may exhibit oedema and cyanosis When the lower limb is involved, permanent pitting oedema may remain (Wilson, 1940). The muscles sometimes waste either immediately or after a variable period. When a limb is affected, its bones may become osteoporotic (see Schönfeld 1929), or show Sudeck's atrophy (Sudeck, 1910) Schleicher (1949) describes chronic inflammatory changes in the marrow of the affected bones. Pleural reactions and haemorrhagic pleural effusions may appear beneath zoster of the chest wall (Curtiu, 1902 ; Mann, 1931) or there may be symptoms and signs of irritation of the parietal peritoneum (Ford 1946)

In cases of ophthalmic herpes sterile inflammatory changes often occur in the eye, resulting in a panophthalmitis and not infrequently in the appearance of haemorrhages and thromboses in the retinal vessels. Afterwards the eyelids may remain permanently oedematous. It is reasonable to suppose that these manifestations in the deeper somatic tissues are produced in a manner analogous to those in the skin—namely, by disturbance in the unmyelinated fibres of the posterior spinal and trigeminal sensory nerve roots

Herpes Zoster Accompanied by Visceral Lesions

Unmyelinated afferent fibres with their cell bodies in the posterior root ganglia run from the viscera uninterruptedly through the autonomic ganglia and white rami communicantes to enter the posterior nerve roots with those from somatic tissues. The posterior nerve roots through which the afferent fibres from the various viscera enter the cord are as follows (Lewis, 1942; White and Smithwick, 1947; Kuntz, 1953):

Heart	C 8–D 4 (?5)
Lungs	D 2–5
Oesophagus	D 4–5
Stomach and duodenum	D 6–8
Liver and gall-bladder	D 7–8 right
Pancreas	D 8 left
Small intestine	D 9–10
Appendix and ascending colon	D 10–L 1
Epididymus	D 10
Ovary, testis, and suprarenal	D 10–11
Bladder fundus	
Kidney	D 11–L 1
Uterine fundus	
Colonic flexure	L 2–3
Sigmoid and rectum	
Cervix	S 2–5
Neck of bladder, prostate, and urethra	

It might be expected that irritation of the unmyelinated afferent fibres in the posterior nerve roots or peripheral nerves, or their cell bodies in the posterior root ganglia would produce changes not only in the skin and somatic tissues but also in the viscera. In his classical paper on herpes zoster Head (1893) did not specifically mention visceral involvement In the literature on herpes zoster, however, there are a number of descriptions of localized visceral disturbances accompanying the skin lesions. I have observed this association in numerous cases, of which Cases 1–8 cited below are typical examples. These cases and those taken from the literature are summarized in the Table.

Case 1 Male, aged 42 Ten days before he attended hospital severe pains began in right side of neck and shoulder, followed after three days by rash over right shoulder and front back, and side of lower part of neck. Three days afterwards he complained of cough, thick mucoid expectoration streaked with blood, and some hoarseness. Examination: Typical painful zosteric eruption in distribution of right C3 and 4 segmental zones with hyperalgesia No abnormal physical signs in other systems. Radiographs of chest normal Laryngoscopy red-

WALLACE, D. M., WALTON, R. J., and SINCLAIR, W. K. (1949). *Brit. J. Urol.*, **21**, 357

WALLACE, D. M., STAPLETON, J. E., and TURNER, R. C. (1952). *Brit. J. Radiol.*, **25**, 421

WALPOLE, A. L., WILLIAMS, M. H. C., and ROBERTS, D. C. (1952). *Brit. J. indust. Med.*, **9**, 255

WALTON, R. J., and SINCLAIR, W. K. (1952). *Brit. med. Bull.*, **8**, 158

round to back in distribution of right D 11-L 1 nerve roots. With onset of rash complete constipation set in. A barium enema showed marked spasm of ascending colon with dilatation of gut, distal to this. Constipation partly relieved by administration of atropine by mouth. With healing of rash marked post-herpetic pain remained, but more normal bowel function gradually returned. Two years after onset of herpes bowels still remained sluggish.

Case 5.—Female, aged 62. Developed herpes in right D 11-L 1 distribution With onset of rash absolute constipation developed and persisted for three weeks, during which time no response to enemata was obtained. Laparotomy performed, but no obstructive lesion found in bowel. The ascending colon was, however, oedematous and engorged, and was excised. Histologically it showed non-specific inflammatory changes in all coats for a distance of 25 cm. Rash faded and the scars healed within six weeks, but pain persisted with some severity for nearly two months. For next 10 years the affected region remained tender and bowel function extremely sluggish. Emptying never occurred without use of purges One month after development of the herpes her two daughters developed chicken-pox.

Case 6.—Female, aged 60 Two weeks previous to attendance at hospital she developed painful irritation of left lower abdomen followed by a herpetic rash passing to loin and back. Two days prior to attendance sudden severe frequency and burning micturition began, and on day of attendance some haematuria occurred She was severely constipated *Examination:* Typical herpetic rash and hyperalgesia in left D 11-12 segmental areas of abdomen and loin Urine. albumin and blood; catheter specimen: numerous R.B.C. and pus cells; no organisms present. Cystoscopy. marked redness and oedema of bladder fundus, chiefly on left side. Barium enema. marked spasm of ascending and transverse colon and dilatation of descending colon. Treated with tincture of hyoscyamus, 30 min. (1.8 ml.) t d s, with some relief of urinary symptoms and constipation. With healing of the skin lesions urinary symptoms and constipation gradually diminished, though both these and pain persisted during the next six months.

Case 7—Male, aged 50. Two months prior to attending hospital he developed severe left lumbar pain spreading into left loin, iliac fossa, and front of thigh Three days later zoster appeared in this area. At the same time he complained of difficulty in defaecation and constipation, considerable frequency of micturition, difficulty in emptying the bladder, and burning pain in penis when bladder was full and during micturition. Symptoms at first severe, but diminished somewhat in severity in course of next two months. Visceral symptoms varied with pain *Examination:* Site of zoster scabs very hyperalgesic and corresponding to left D 12-L 2 segmental areas Cystoscopy· marked congestion and submucous haemorrhages in the floor and root of the bladder mucosa, which bled easily and appeared inflamed. Urine: no organisms, but some pus cells present Barium enema. markedly spastic descending colon and rectum, with dilatation of transverse colon above (FIG. 3) Cerebrospinal fluid· normal Repetition of barium enema four weeks later showed much diminution in spasm. Over the next two years he was subject to bouts of pain with inability to empty the bladder.

Case 8 — Male, aged 52. Two weeks previous to attending hospital he developed pain in front of left thigh and knee, extending to outer side of left hip Four days later a rash appeared in painful area and after another four days he developed severe constipation with a desire to empty the bowels; he passed dark blood-stained mucus and had some difficulty and pain on micturition *Examination:* Typical herpes zoster and hyperalgesia in

FIG. 3.—Case 7. Left D 12-L 2 zoster. Barium enema radiograph showing spasm of descending colon

left L 2-3 segmental areas. Urine: pus cells but no organisms Rectal examination: normal. Sigmoidoscopy: swollen reddened mucosa at uppermost limit to which the instrument could be passed. Barium enema: severe spasm of descending colon Cystoscopy: neck of bladder intensely red and oedematous. Intestinal and urinary symptoms considerably relieved by administration of tincture of hyoscyamus, 30 min. (1.8 ml.) t d s Pain, skin lesions, and intestinal disturbance gradually disappeared in the next three months.

Comment

After reference to the course of the afferent visceral fibres shown in the Table it will be observed that the visceral disturbance in cases of herpes zoster occurs particularly in those viscera, or parts of viscera, supplied with afferent fibres by the posterior nerve roots corresponding to the zonal skin areas affected As in the skin and deep tissues, so the effects on the viscera are to produce vasodilatation, oedema, haemorrhages, and inflammatory changes, and to predispose to vascular thromboses The affected gut exhibits local hypermotility and spasm. The evidence suggests that, apart from the parasympathetic and sympathetic, the viscera receive a third type of nerve supply which may affect their activity. The bipolar cell bodies lie in the posterior root ganglia, and their peripheral axons pass to the viscera without relay through the autonomic ganglia. They are, in fact, the so-called visceral "afferent " fibres. This perhaps explains to some extent the frequently reported variable and uncertain effects on the viscera of stimulating the rami communicantes of the spinal nerves and the sympathetic chain in which such fibres run. It further raises the problem of whether "antidromic" centrifugal impulses are normally conducted by the "afferent" unmyelinated fibres

Summary

In cases of herpes zoster the inflammatory skin lesions are thought to result from disturbance of the ganglion cells of the posterior or trigeminal root ganglia or of the "afferent" fibres of the posterior nerve roots or the peripheral nerves. Similar nerve fibres originate or end in the viscera. Evidence is adduced which seems to indicate that, accompanying the segmental skin lesions, there occurs disturbance of the viscus, or part of the viscus, innervated by the corresponding nerve roots. which results in inflammatory lesions and spasm in the case of the hollow organs. This suggests that, in addition to the sympathetic and parasympathetic pathways, there exists a third nerve pathway by which visceral activity may be altered.

REFERENCES

Andréassian (1934). *Paris méd*, 2, 533
Belgodere, G (1924) *Ann. Mal vénér*, 19 813
Bernhardt, M (1890) *Berl. klin. Wschr.*, 27, 695
Bickelonne, H. (1907) *Bull méd (Paris)*, 21, 277
Blechowska, M. (1914) In M H. Lewandowsky's *Handbuch der Neurologie* 5, 316 Springer, Berlin
Brain, W R (1931) *British Medical Journal* 1, 81
—— (1952) Ibid 1, 632
Chesterman, C C (1932) Ibid 1, 563
Clark, D, Hughes, J., and Gasser, H S (1935) *Amer J Physiol*. 114, 69
Curtis, R. G (1922) *Amer J med Sci*, 123, 264

and oedema of right vocal cord Bronchoscopy: marked
...ema and redness,
..tly limited to right
... of tracheal mucosa,
..nding downwards to
...na. With fading of
...skin lesion the cough,
...ectoration, and hoarse-
... gradually ceased.

Case 2.—Female, aged
... Three weeks before
...ming to hospital s h e
...eloped hemigirdle pain
...the right lower chest
...between the shoulder-
...des. Four days later
...ter appeared in this
...ion with a sensation of
...sternal pressure and
...gastric aching immedi-
...ly on swallowing, as
...ough food was stuck.

Fig. 1—Case 2. Right D 6–8 zoster. Barium meal radiograph showing spasm of pyloric antrum

... aching was not improved by taking alkalis. *Examination:*

... spasm had disappeared radiologically. Fractional test meal
... showed some fall in acid secretion.

Author	Site of Zoster	Visceral Disturbance
...ase 1, present series	C 3–4	Redness, oedema, inflammatory changes, and bleeding on right side of larynx and trachea
...ndréassian (1934)	L. cervico-scapulo-thoracic	Endocarditis and tachyarrhythmia
...urner (1909)	D 2	Paroxysmal tachycardia during and following attack
...ann (1931)	D 3–4	Marked bradycardia
...ase 2 of Spillane and White (1939)	C 8–D 2, and D 4–6	Followed 3½ years later by progressively severe anginal pain at site of zoster; cardiac enlargement, and E.C.G. changes
...urtin (1902)	D 7–8	Pleuritic pain, friction rub or pleural effusion
...ann (1931)	D 7–8	
...rain (1931)	D 7–8	
...yle (1932)	D 3–4	Gastro-intestinal disturbance
...arcot (1907)	Intercostal	Disturbance of passage of food down oesophagus
...urtin (1902)	Upper abdominal	Anorexia and constipation
"	Lower thoracic margin	Bowel atony and abdominal distension
...tayer (1935)	Abdominal	Gastro-intestinal disturbance preceding, during, and following rash
...rlach (1908)	"	"
...ielschowsky (1914)	"	"
...uchs (1840)	"	"
...ase 2, present series	D 6–8	Abdominal colic
...Case 3, present series	D 7–8	Severe spasm of prepyloric region of stomach (Fig. 1) with reddening of gastric mucosa, symptoms being partly relieved by atropine
		Nausea, vomiting, and anorexia, followed by haematemesis and appearance of gastric ulcer on lesser curvature (Fig. 2)
Hess and Faltischek (1925)	D 6–10	Gastric hyperacidity cured by paravertebral sympathetic block
Stone (1942)	D 9–10	Haematemesis
Fitzpatrick (1948)	D 9–10	Nausea, anorexia, burning epigastric sensation, haematemesis, and achlorhydria Radiographs showed no peptic ulcer Symptoms unrelieved by alkalis and gradually disappeared with skin eruptions
" Surgeon " (1950)	D 8–10	Intestinal paralysis and vomiting Colon radiologically normal, the disturbance presumably being in the small intestine"
Donaldson (1948)	D 9–11	Anorexia, nausea, and haematemesis, the symptoms disappearing with the skin changes
Curtin (1902)	D 10–11	Fever and symptoms resembling appendicitis
Case 4, present series	D 11–L 1	Intense constipation and local spasm of ascending colon with dilatation distal to this, symptoms being partly relieved by atropine
Case 5, present series	D 11–L 1	Intense constipation, though taking no drugs Laparotomy revealed inflammatory changes in ascending colon

Author	Site of Zoster	Visceral Disturbance
Curtin (1902)	Over kidney and passing to abdomen	Persistent haematuria
Grossmann (1932)	D 10–L 2	Renal colic No urinary disturbance or evidence of calculi
Van der Scheer (1913)	D 12	Polyuria and severe persistent constipation
Nieuwenhuijse (1914)	D 12	Ileus
Case 6, present series	D 11–L 2	Spasm of ascending and transverse colon and dilatation of distal colon. Inflammatory changes in bladder mucosa, especially on left Symptoms partly relieved by hyoscyamus
Darget (1929)	Inguinal	Cystitis and haematuria Cystoscopy showed a raised inflammatory area on bladder wall
Volk, quoted by Schönfeld (1929)	Buttock	Zoster of bladder and haemorrhage cystitis
Ford (1946)	Abdominal	Symptoms of bladder irritation
Watts (1948)	Upper lumbar root zone	Disturbance of bladder function and haematuria
Siding (1909)	L 1	Rectal and bladder disturbance
Case 7, present series	D 12–L 2	Transient severe inflammatory changes in bladder mucosa. Spasm of descending colon and rectum and dilatation of transverse colon above (Fig 3)
Case 8, present series	L 2–3	Inflammation of mucosa of sigmoid colon, spasm of descending colon (Fig 4). Inflammation of neck of bladder Symptoms partly relieved by hyoscyamus
Dubois (1926)	Buttock	Frequency, dysuria, and eventually urinary retention. Cystoscopy showed vesicles on left side of base and lateral wall of bladder
Wolbart, quoted by Dubois (1926)	Buttock	Herpes of ureteric orifice
Satani (1926)	"	Zoster of bladder on same side and ...
Rouyer (190...	"	
Chesterman	"	
Belgodere (1...	"	
Davidsohn	"	
Bernhardt (1890)	"	rectum
Oppenheim (1911)	"	
Tomey (1909)	Sacral	Frequency and disturbance of bowel emptying
Parsat (1910)	Gluteal region, scrotum, and perineum	Urinary and faecal retention for 14 days
Frankl-Hochwart (1913)	R genital area	Dysuria and disturbance of bowel emptying
Spitzee (1925 6)	Buttock and una leg	Severe urinary disturbance lasting 3 weeks
Mann (1931)	Sacral	Rectal and bladder disturbance

Case 3.—Male, aged 72 No previous digestive disturbance.
He developed herpes zoster of right D 7 and 8 zonal areas. At
height of attack he complained of nausea, vomiting, and severe
anorexia, which lasted for several months. Skin lesions, followed
by post-herpetic pain of two types, a constant ache, and a
sharp stabbing pain
brought on by move-
ment of trunk ; pain
varied in severity,
but worse in damp
a n d cold weather.
T w o months after
onset two severe
haematemeses, each
of about 1 pint (570
ml) A barium meal
one m o n t h later
showed an ulcer
crater on the lesser
curvature o f t h e
incisura (Fig. 2).
Despite various
antacids the diges-
tive symptoms per-
sisted.

Fig. 2.—Case 3. Radiograph taken during barium meal, showing gastric ulcer on lesser curvature developing with zoster of right D 7–8 zonal areas

Case 4. — Male,
aged 63. Three
weeks previous to
e x a m i n a t i o n he
noticed a numb sen-
sation and *tenderness* in region of right hip when he lay on it,
and a week later this region became painful. Within a few days
a segmental zosteric eruption appeared in the painful region,
extending from 1½ in. (3.8 cm.) below the umbilicus to groin and

MALIGNANT CHANGE FOLLOWING HERPES SIMPLEX

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In 1955 I described a series of cases in which herpes zoster of the skin or breast was followed after a variable period by malignant change in the affected tissues (Wyburn-Mason, 1955). During the investigations at the Royal Marsden Hospital there was also observed a number of cases of malignant change in the skin of the lip which were preceded by herpes simplex. No mention of herpes simplex as a premalignant condition has been found in an exhaustive search of the literature. A brief account of six of these cases follows.

Case Reports

Case 1.—Man aged 65. For five years he had been subject to recurrent herpes at the right angle of the mouth. After the last attack three months prior to hospital attendance a lesion had persisted, gradually increased in size, and bled from time to time. On examination a circular raised ulcer with everted edge was present, measuring 1.5 cm. in diameter (Fig 1) Biopsy showed the lesion to be a squamous carcinoma.

Case 2.—Man aged 72. Three years previous to hospital attendance he developed herpes of the left upper lip. This was followed by the formation of a wart and by recurrent herpetic attacks in the same area After the last attack, four months previous to examination, a growth had appeared (Fig 2) and biopsy showed the presence of a squamous carcinoma.

Case 3.—Man aged 62 Eight weeks prior to attendance he developed herpes of the right upper lip, which almost healed within a fortnight, but then broke down again and ulcerated, gradually increasing in size and bleeding at intervals The appearance is shown in Fig. 3. Biopsy showed the lesion to be a squamous carcinoma.

Case 4.—Woman aged 67 Seven weeks prior to attendance she developed a severe coryza, during which there appeared extensive herpes of the lower lip and the right

FIG. 1—Case 1 Squamous carcinoma arising in lesions of herpes simplex

angle of the mouth spreading on to the cheek. The lesion healed within a fortnight, except for a small area near the angle of the mouth, and during the next four weeks this rapidly increased in size and began to bleed. Biopsy showed the presence of a squamous carcinoma.

Case 5.—Woman aged 65. Eighteen months prior to hospital attendance she developed herpes of the left lower lip, which continually healed and broke down, particularly after exposure to sunlight. After such an exposure some three months prior to hospital attendance the lesion appeared, failed to heal, and then rapidly increased in size and began to bleed. It was found on biopsy to be a squamous carcinoma. She was treated with x rays with complete healing Eighteen months later, after a further exposure to sunlight, she developed a fresh herpes in the same area.

Case 6.—Man aged 65. A year previous to attendance he pulled a piece off the lower lip with a cigarette. Next day herpes appeared in the denuded area and spread to involve the entire mucocutaneous margin of the lower lip This was followed by crusting and bleeding after mild trauma, but never by complete healing (Fig 4) Biopsy a year after the onset showed the presence of a squamous carcinoma. He was treated by x rays with complete healing. A year later, after exposure to sunlight, he developed a further herpetic lesion in the original area

Discussion

The six patients were aged between 60 and 70 years at the time of onset of the herpetic lesion One (Case 2) developed a wart, and two (Cases 5 and 6) showed recurrence of the herpes when exposed to sunlight. Such an abnormal sensitivity to sunlight is also observed in the skin of tar-workers, those exposed to x rays and arsenic, and in cases of xeroderma pigmentosa, in all of which malignant change may also occur in the affected skin. The cases described are of more than academic interest, as they might serve as evidence of the virus theory of cancer causation. It is important to consider certain observations about herpes simplex (see Van Rooyen and Rhodes, 1948 ; Stoker, 1957).

The pathological changes of herpes simplex or zoster and herpetiform lesions are those of an inflammation with oedema between the epidermal cells, and there is nothing specific about the changes. Herpes simplex and herpetiform lesions may be produced by many different factors, which include general infections or intoxications ; respiratory infections ; the administration of drugs, such as arsenic, antigenic substances, or antisera ; the local application of irritant (blistering) agents , or chronic exposure to tar, x rays, etc. They may also occur on skin which is the site of causalgic pain. In cases of herpes zoster the same type of lesion is produced as a result of disturbance of the innervation of the affected part The virus of herpes simplex is the causative agent not only of herpes on the lips or nostrils, but also of herpes genitalis and cornealis, herpetic (aphthous, Vincent's, or ulcerative) stomatitis, eczema herpeticum (a type of Kaposi's varicelliform eruption), and meningo-encephalitis The last three conditions do not recur, but many individuals are subject throughout life to recurrent attacks of herpes around the lips and nose, or of the genitalia, or of the cornea, and these develop in response to non-specific stimuli, such as sunburn, fever, or trauma and not through exposure to another source of herpes virus. In women, recurrence is often associated with the menstrual periods.

The vesicle fluid from herpes simplex lesions affecting the face, genitalia, or cornea may produce herpetic vesicles when inoculated into the skin or keratitis when applied to the conjunctiva of the rabbit, and it is therefore usually considered that all simple herpetic lesions are due to the same

Darget, R. (1929) J. Urol. méd chir., 27. 229.
Davidsohn, H. (1890) Berl. klin. Wschr., 27, 695
Donaldson, E. D T. (1948) British Medical Journal, 2, 839
Dubois, F. E. (1926) J Urol., 15 583.
Feindel, W H., Weddell, G and Sinclair, D C. (1948) J Neurol
    Neurosurg. Psychiat , 11 113
Fitzpatrick, P E (1948) British Medical Journal, 1 1206
Ford, F. R. (1946)
    and Adolescenc
v Frankl-Hochwart
Frisch, A (1908)
Fuchs, C H, (1840
    Anhänge in n
    Göttingen.
Fulton, J F (1949) Physiology of the Nervous System, 3rd ed   Oxford
    Univ Press London
Grossmann, W (1932) Klin Wschr , 11, 1181.
C   ,   C   and   Proc   ol (Paris) 535
                              med Hôp Paris 36, 437

                              Klin , 21, 1683.
                              vous System, 4th ed   Lea and
                              Masson.
                              Rec , 71, 387,
                              ses of the Human Skin Shaw
    London
—— (1942) Pain Macmillan. London
Mann, W. (1931) Z klin Med , 118, 630

Nieuwenhuijse, P. (1914) Z. ges Neurol Psychiat , 32, 45.
Oppenheim, H (1911). Textbook of Nervous Diseases, translated by A
    Bruce, vol. 1. Daren Press, Edinburgh
Parsat (1910) Ann Derm Syph. (Paris), 1, 332
Ramond, L. (1923) Progr. méd (Paris), 38 97.
Raason, S W   Di   H V
Rayer, P. (193
Rouyer, E. (19
Ryle, J A (19
Sassati (1926)
Van der Scher
Schleicher, E.
Schönfeld W
    edited by
Sidney, A (19
Spillane, J D
Spitzer, R (1
Stone, R. V (
Sudeck (1910)
    Springer.
                                              20, 9
                                              rnal, 2, 1026
                                              (s), 9, 297
                                              Journal, 1, 175
                                              The Autonomic Nervous System,

Wilson S A K (1940) Neurology Arnold, London

IMPROVED SCINTILLATING MEDIA FOR RADIATION DOSIMETRY

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THE use of scintillation detectors for X and γ-radiation dosimetry, particularly at lower quantum energies, is limited by the energy-dependence of the response of the luminescent medium. Instruments incorporating crystalline anthracene or plastic polymers containing p-terphenyl as the scintillating medium have at quantum energies down to 250 keV, but as was shown by Belcher (1953) the sensitivity of such

Ter-Pogossian (1952), Brucker (1952), Belcher (1953), Carr and Hine (1953) and others.

Many attempts have been made to extend the working range of scintillation detectors for dosimetry below 250 keV and the problem has recently been reviewed by Rosman and Zimmer (1956). Breitling and Glocker (1952) and Glocker and Breitling (1952) have described the use of an air-equivalent liquid scintillator consisting of a solution

FIG. 1. FIG. 2. FIG. 3.
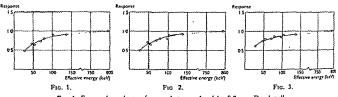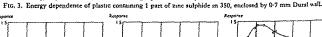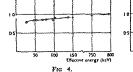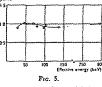
FIG. 1. Energy dependence of pure plastic, enclosed by 0·7 mm Dural wall.
FIG. 2. Energy dependence of plastic containing 1 part of zinc sulphide in 500, enclosed by 0·7 mm Dural wall.
FIG. 3. Energy dependence of plastic containing 1 part of zinc sulphide in 350, enclosed by 0·7 mm Dural wall.

FIG. 4. FIG. 5. FIG. 6.
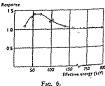
FIG. 4. Energy dependence of plastic containing 1 part of zinc sulphide in 300, enclosed by 0·7 mm Dural wall.
FIG. 5. Energy dependence of plastic containing 1 part of zinc sulphide in 250, enclosed by 0·7 mm Dural wall.
FIG. 6. Energy dependence of plastic containing 1 part of zinc sulphide in 200, enclosed by 0·7 mm Dural wall.

devices as calibrated against an air-wall ionization chamber falls at lower energies. This fall is mainly due to the low mean atomic number of these media compared with air, but may also in part be attributed to lack of proportionality between energy absorption and light emission by the luminophor and in part to absorption of radiation by the enclosing metal wall. The principles governing this variation of sensitivity with energy have been discussed by Ittner and

of p-terphenyl in a mixture of benzene and chlorobenzene, and of polycrystalline screens of mixed phenanthrene and chlorophenanthrene for this purpose. Ittner and Ter-Pogossian (1952) and Cole, Duffy, Hayes, Lusby and Webb (1952) have used compound crystals of anthracene and calcium tungstate and suspensions of ground calcium tungstate in polyester plastic. None of these materials, however, provides a completely satisfactory solution

virus. There is, however, some doubt whether a virus can be demonstrated in all herpetic simplex lesions. According to Stoker, " there is no doubt that the virus is present during each of these recurrent attacks, because it can invariably be isolated in the early stages." It has been suggested (Burnet and Williams, 1939) that in recurrent cases herpetic infection occurs in childhood and persists throughout life. This is based on serological and skin tests, the sera of most adults containing antibodies against the virus. It is supposed that the virus remains within the epidermal cells, dividing with them, and normally causing no damage. A number of non-specific stimuli which lead to cell breakdown may release the virus particles

Stalder and Zurukzoglu (1936) showed, however, that areas of facial skin subject to recurrent herpes no longer developed the disease if transplanted to another part of the body Yet a recurrent attack usually occurs in roughly the same site. Because of this some favour the central nervous system, dorsal root ganglia, or cutaneous nerve endings

of what may occur, especially in elderly subjects, at the site of any chronic irritation or inflammation, such as eczema or psoriasis, and not the specific result of virus infection. On the other hand, if all, and particularly recurrent, cases of herpes simplex are due to local infection with herpes simplex virus, and since the subjects may exhibit abnormal sensitivity to sunlight as do tar-workers and those subject to x rays or arsenical dermatitis and xeroderma pigmentosa, it may be that infection by the virus, which is widespread, is an important cause of malignancy. Since the buccal mucosa and the skin of the genitalia and other tissues may also be infected by herpes simplex there is a possibility that in some cases in these tissues, too, the infection may be related to the later appearance of malignancy.

Summary

Six cases of herpes simplex of the lips in old people followed by the development of squamous carcinoma

FIG. 2.—Case 2

FIG. 3.—Case 3.

FIG. 4.—Case 6

as the permanent home of the virus. In this respect it will be recalled that the lesions of herpes zoster, which are due to infection with the herpetic zoster virus, are identical pathologically with those of herpes simplex, but the causative lesion for the skin change is found in the posterior nerve root ganglia (Lewis, 1927). It is thus by no means certain that the lesions of herpes simplex are always due to, or contain, a virus affecting the skin. It seems possible that this type of tissue reaction in the skin may result from various forms of irritation, of which the herpes simplex virus is one example ; that even in recurrent cases of herpes simplex local irritation plays a part in causing the recurrence, and that, as in cases of herpes zoster, disturbance of local nerve fibres may be a factor in causing this.

Whatever the explanation of recurrent herpes simplex, it seems that the appearance of the original lesion may predispose to malignant change in elderly subjects. Malignant change following herpes simplex may simply be an example

six weeks to five years afterwards are described. The significance of the observation in relation to the virus theory of cancer causation is briefly considered.

REFERENCES

Burnet, F. M., and Williams, S W. (1939) *Med. J Aust .* 1, 637.
Lewis, T. (1927). *Blood Vessels of the Human Skin and Their Responses* Shaw, London.
Van Rooyen, C. E., and Rhodes, A. J (1948). *Virus Diseases of Man.* 2nd ed Nelson, New York.
Stalder, W., and Zurukzoglu, S. T. (1936) *Zbl. Bakt., I. Abt Orig.,* 136 94
Stoker. M G R. (1957) *British Medical Journal,* 1, 963.
Wyburn-Mason. R. (1955). *Ibid.,* 2, 1106

Irradiation in all cases was made at right angles to the probe axis, and correction was made where necessary for the direct effect of radiation on the photomultiplier tube.

It will be observed that as the proportion of zinc sulphide is increased, the falling response at low energies due to the low mean atomic number of the pure plastic is progressively corrected. A mixture containing 1 part in 300 by weight of zinc sulphide has a response which falls only slowly with energy down to effective energy of 30 keV. The variation of sensitivity of this mixture with energy is less than ± 10 per cent over the range 30 keV to 1 MeV and measurements on a number of batches showed that this behaviour is accurately reproducible.

Effect of probe dimensions and composition of capsule wall

At energies below 30 keV, the form of the energy-response curve is determined very largely by the nature of the capsule wall. Figure 8 shows the results of studies over the lower energy range using the 1/300 mixture firstly with a 0·7 mm Dural wall, and secondly with a wall consisting of a 0·01 mm aluminium foil covered with 10 mg/cm² of black paper. Measurements in the latter case were made inside a Perspex build-up device to ensure electronic equilibrium. It will be seen that by using an extremely thin wall of approximately air-equivalent material, the working range can be extended at least as far as 20 keV. Figure 9 shows similar measurements with the 1/250 mixture.

With a given mixture and wall thickness, performance is not greatly affected by probe dimensions. This is illustrated by Fig. 10, which shows the results of measurements with the 1/300 mixture incorporated into probes of external diameter 6 mm, 8 mm and 12 mm, all with 0·7 mm Dural wall.

Effect of composition on luminescent efficiency

Table I shows the luminescent efficiencies of the various mixtures, expressed in terms of the responses

TABLE I

Composition	Response to Ra γ radiation	Composition	Response to Ra γ radiation
Pure plastic	1·00	1/300 Zn S	0 83
1/500 Zn S	1·05	1/250 Zn S	0 90
1/400 Zn S	0 85	1/200 Zn S	0 82
1/350 Zn S	0 89	—	—

obtained with radium γ radiation, the response of the pure polystyrene/p-terphenyl/tetraphenylbutadiene plastic being taken as unity. The mixtures compare favourably with the pure plastic in light output.

SUMMARY

An improved scintillating medium for radiation dosimetry with energy response varying by less than ± 10 per cent over the energy range 20 keV to 2 MeV has been developed. The ... of this material compares favourably with that of the pure plastic. The effects of probe dimensions and wall composition on the performance of probe-type dose-rate meters incorporating this material are described.

REFERENCES

BELCHER, E. H., *Brit. J. Radiol.*, 1953, xxvi, 455.
BREITLING, G, and GLOCKER, R, *Naturwissenschaften*, 1952, xxxix, 84.
BRUCKER, G. J., *Nucleonics*, 1952, x(xi), 72.
CANN, B. T., and HEES, C. I., *Nucleonics*, 1953, xi(vi), 53.

... lxxxviii, ...
ITTNER, W. B, and TER-POGOSSIAN, M., *Nucleonics*, 1952, x(ii), 48.
ROSMAN, I. M., and ZIMMER, K. G., *Z. Naturforsch*, 1956, xib, 46.

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to the problem. The ideal scintillating medium for radiation dosimetry should be a stable homogeneous solid, easily machined, of high transparency and luminescent efficiency and should have a

FIG. 7.
Diagram of capsule.

Effect of composition on energy response

Figure 1 shows the results of studies of the variation in sensitivity per röntgen with energy of a probe-type scintillation dose-rate meter using the pure plastic as scintillating medium. Figures 2 to 6 show similar results with mixed media containing different proportions by weight of zinc sulphide. The studies were made with a dose-rate meter of the type described by Belcher (1953), using a Dural probe of 6 mm outside diameter. The media were mounted in interchangeable Dural capsules of wall thickness 0·7 mm, forming the terminal 1·5 cm of the probe (Fig. 7).

FIG. 8.   FIG. 9.   FIG. 10

FIG. 8. Energy dependence of plastic containing 1 part of zinc sulphide in 300 enclosed by walls of different composition.
O 0 7 mm Dural wall.
X 0 01 mm Al + 10 mg/cm² paper wall.

FIG. 9. Energy dependence of plastic containing 1 part of zinc sulphide in 250 enclosed by walls of different composition.
O 0 7 mm Dural wall.
X 0 01 mm Al + 10 mg/cm² paper wall.

FIG. 10. Energy dependence of plastic containing 1 part of zinc sulphide in 300 incorporated in probes of different external diameters.
O 6 mm O.D.   X 8 mm O.D.   + 12 mm O D.

response independent of energy over the entire working range, offer advantages over media hitherto available for dosimetric work. These materials consist of polystyrene/p-terphenyl/tetraphenyl-butadiene plastic containing an admixture of finely dispersed silver-activated zinc sulphide. The proportions of zinc sulphide used are not sufficient markedly to impair the transparency of the plastic matrix and whilst the resulting mixtures are not truly homogeneous and would thus be unsuitable for counting applications, they are nevertheless satisfactory for dosimetric use. They are readily machined and polished, and have luminescent efficiencies comparable with that of the pure polystyrene/p-terphenyl/tetraphenylbutadiene plastic.

We have developed* mixed scintillating media which, while they do not completely satisfy all these requirements, offer advantages over media hitherto

Measurements were made by comparison with a Baldwin-Farmer ionization chamber dosemeter, using radium γ radiation (mean quantum energy 0·79 MeV) and the following sources of X radiation:

	kVp	Inherent	Added	Effective energy keV
1.	250	2·5 mm Al	0·6 mm Sn / 0 25 mm Cu / 1·0 mm Al	137
2.	250	2·5 mm Al	0·5 mm Cu / 1·0 mm Al	103
3.	250	2·5 mm Al	4·0 mm Al	79
4.	200	2·5 mm Al	1·0 mm Cu / 1·0 mm Al	97
5.	200	2 5 mm Al	4·0 mm Al	70
6.	200	2.5 mm Al	None	47
7.	140	1·0 mm Al	0·2 mm Cu / 1 0 mm Al	54
8.	100	1·0 mm Al	1·0 mm Al	28
9.	50	1·0 mm Al	1·0 mm Be	21·5
10.	43	0·7 mm Al	1·0 mm Be	18·6
11.	29	0·4 mm Al	1·0 mm Be	14·0

The process of ionization and excitation in an atom, or the attachment to an atom of a released electron, leads to altered chemical properties of the molecules of which these atoms are part, and are the start of the chain of chemical changes which lead to the observed biological damage.

The spatial distribution of ionizations and excitations influences the concentration of the initial chemical change, and therefore affects the severity and type of biological effect produced. One parameter to be considered in the analysis of the biological change produced by a given type of radiation is the "specific ionization" or "linear ion density," which is the average value of the number of ions produced per unit distance along the tracks of the ionizing particles. The lowest values of specific ionization (the least dense ionization tracks) are produced by high energy electromagnetic radiation. In general the lower the energy of the electromagnetic radiation, the greater the value of the specific ionization. The highest values of specific ionization are given by the particulate radiations such as protons, α-particles, and also fast neutrons which in soft tissue ionize by virtue mainly of ejected protons. Table I (Gray, 1947) gives approximate values for the specific ionization of certain radiations, in number of ions per micron of path in unit density material.

TABLE I

VALUES OF SPECIFIC IONIZATION

(Gray, 1947)

Radiation					Specific ionization (ions per micron)
γ-rays from radium	..	.	..	..	11
X-rays produced at 1,000 kV	..	..	.	..	13
X-rays produced at 200 kV	..	..	.	..	80
Neutrons of energy 12 MeV	.	..	,.	..	290
Neutrons of energy 0 4 MeV	.	..	..	..	1,100
α-particles from natural disintegration of radium	.				3,700

Units of radiation dosage

The unit of dose for x- and γ-radiation employed in recent years has been the roentgen ("r"), which is measured by the ionization produced in a given mass of air under certain defined equilibrium conditions. Although this unit is of great service it has certain serious limitations, in that it *does not give a measure of the energy absorbed in the tissue* when the tissue has an effective atomic number appreciably different from that of air or water. Also the roentgen cannot be applied directly in the measurement of the dosage of particulate radiations such as neutron beams.

To avoid these difficulties other units are sometimes used. In many ways the most satisfactory of all units is the recently defined "rad," which represents an energy absorption of 100 ergs per gramme. This poses new problems, not of definition, but of practical measurement and has not yet come into general use. In the case of soft tissue, one rad of ionizing radiation will represent an energy absorption some 7 per cent greater than that for one roentgen.

The unit of dosage used most frequently in recent years when dealing with particulate radiations has been the "rep" (roentgen equivalent physical). So far as soft tissues are concerned the roentgen and rep can be considered to represent the absorption of the same amount of energy per unit mass.

CARCINOGENIC EFFECTS OF RADIATION

A. GLUCKSMANN, L. F. LAMERTON AND W. V. MAYNEORD

INTRODUCTION

The problem of radiation carcinogenesis is not merely one of demonstrating that ionizing radiation can produce malignancies in certain tissues of various species of animals, but of determining the antecedents of tumour production in terms of radiation dose, dosage rate, volume of tissue irradiated, predisposing tissue conditions, and other factors. Only with adequate data relating to these variables can the fundamental question relating to mechanisms of radiation carcinogenesis be decided, such as the relative importance, under given conditions, of the direct and indirect effects of radiation.

For obvious reasons the least well documented of the data is that referring to radiation-induced cancer in man. Nevertheless the data that exist are of the greatest importance and an attempt is made in this chapter to give a reasonably full review of the reported human data. This is followed by a survey in some detail of certain of the animal experiments which have a bearing on the problems that have arisen in consideration of the human data.

The radiations considered here are the high energy electromagnetic radiations (x-rays and γ-rays) and the particulate radiations such as electrons and neutrons. The terms x-rays and γ-rays are both applied to high energy electromagnetic radiation, and the definition employed is that x-rays are those produced by high energy devices (x-ray tubes, betatrons, linear accelerators, and so on) while γ-rays are the electromagnetic radiations produced directly from atomic nuclei during the process of disintegration.

Before reviewing the literature it will be convenient to discuss briefly certain physical aspects of the interaction between radiation and matter.

Interaction between radiation and matter

The passage of high energy electromagnetic or particulate radiation through matter is marked by changes in certain of the atoms of the material close to the path of the quanta or particles comprising the beam of radiation. For x-rays and γ-rays and for electrons these changes will be the result of interactions with the orbital electrons of atoms, either by ionization, when an electron is removed from the atom, or by excitation, when an electron is displaced to a new energy level within the atom. Neutrons, on the other hand, interact with atomic nuclei to produce recoil movement of the nucleus which may be sufficient to produce dense ionization and excitation in surrounding atoms of the material, and under certain conditions leading to nuclear changes which result in the emission of γ-rays and occasionally α-particles or protons. The newly-formed nucleus may itself be radioactive and contribute to the irradiation of the tissue.

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patients treated by x-rays for ankylosing spondylitis, which may possibly be due to the carcinogenic action of radiation (Court Brown and Abbatt, 1955).

The following are the main sites for which there is evidence of radiation-induced cancer in man:

(1) *The skin* —Cancer of the skin may follow external radiation, incurred as an occupational hazard, or following diagnostic or therapeutic procedures for deep-seated conditions or for benign dermatoses.

(2) *The bones.*—Osteogenic sarcomas have been observed after external irradiation for tuberculous or other osteomyelitic conditions or after the ingestion of radioactive salts which are finally deposited in the bone matrix, as for instance in the case of the dial painters.

(3) *The blood forming organs.*—These may react to external irradiation with the production of leukaemias.

(4) *The lungs.*—Cancer may occur after inhalation of radioactive dust or gas, as in the case of the uranium miners.

Occasional radiation-induced tumours have been reported at other sites, including the pharynx, thyroid, antrum, liver, ovary and the uterus.

The incidence, histological types and precursory lesions of cancers caused or contributed to by radiation will now be described under the various sites before the general problem of carcinogenesis due to radiation is considered and related to experimental work.

Skin tumours

Introduction

Skin cancers have been found in the irradiated region of patients treated for deeper-seated malignant conditions or for benign dermatoses. A clear distinction should be made between those cancers appearing in a previously normal skin region and those following irradiation of skin lesions such as lupus or psoriasis which may themselves predispose to carcinomatous change. In the first group irradiation may be the main cause of malignancy while in the second group it may be only a contributory cause, and it is possible that the mechanism of carcinogenesis is different in the two groups.

Reviews of reported cases of skin cancers following therapeutic and, more rarely, diagnostic radiation procedures have been made by Holthusen and Englmann (1931), Grützmacher (1942) and others.

Incidence of histological types

The histological features of skin cancers are the same whether they occur spontaneously or are produced by radiation. There is, however, a difference in the proportion of squamous-cell and basal-cell carcinomas in the spontaneous and radiation-induced group The combined data of Lacassagne (1945a), Schrek (quoted from Moore, 1945) and Harnett (1952), show that 63 per cent of 5,237 spontaneous skin cancers were of the basal type, while of 111 patients with radiation-induced cancers, 102 had squamous-cell cancer and 2 had basal-cell cancer and 2 had both (data from Lacassagne, 1945a, Holthusen and Englmann, 1931, Grützmacher, 1942, and Teloh, Mason and Wheelock, 1950).

In a review on the carcinogenic effects of radiation a common dosage unit is not possible, since many authors have not provided the relevant data for a conversion from their units

The radiation dose delivered to a tissue containing a radioactive substance can be calculated from a knowledge of the concentration of the isotope in terms of microcuries per gramme of tissue (Mayneord, 1950). The calculation is straight-forward if the activity is uniformly distributed in the tissue and the linear dimensions of the tissue are large compared with the range of the radiation emitted. If these conditions are not satisfied the calculation of the dose demands also precise knowledge of the dimensions of the tissue considered and the degree of non-uniformity of the activity.

Relative biological efficiency (R.B.E.)

Since the type and degree of biological change is affected by specific ionization, and also by other physical variables in addition to the dose, it follows that the same dose of two different types of radiation may have different effects. To compare the effectiveness of two types of radiation the term "relative biological efficiency" or "R B.E." is used, which is defined as the inverse ratio of the doses necessary to produce a given effect. The R.B.E. of two radiations may itself vary with the type of damage considered. Thus, the R.B.E. of α-radiation to γ-radiation is about 10 for certain types of chromosome damage, whereas it is unity, or less, for some cytoplasmic changes.

RADIATION-INDUCED CANCER IN MAN

Introduction

The first case of x-ray cancer was described by Frieben in Hamburg in 1902, in a man aged 33 years, who had been employed in the manufacture of x-ray tubes and tested the apparatus with his hand. A marked radiation dermatitis developed on the dorsal surface of his hand and after 3 years small persistent ulcers appeared. The edges became cancerous, and the dorsum of the hand was eventually completely ulcerated. The cubital and axillary lymph nodes contained metastases; the arm was, therefore, disarticulated at the shoulder.

Numerous other cases of chronic radiodermatitis particularly on the hands of radiological workers and those employed in the manufacture and sale of x-ray apparatus were observed in the early years of this century and the tendency to malignant change of these chronic skin lesions was recognized. Hesse in 1911 described 50 cases of cancer developing in previously normal skin, in persons exposed occupationally to radiation, and in 4 patients following radiation treatment.

The carcinogenic risk to man from ingested radioactive material is demonstrated by the osteosarcomas occurring in the group of dial painters working in a New Jersey factory between 1917 and 1924. A number of bone tumours following radiation procedures have also been reported, a high proportion occurring in areas of infected bone. The "Bergkrankheit" of miners in the Schneeberg and Joachimsthal mines has been found to be a lung cancer possibly attributable to radiations emitted from inhaled radioactive dust and gas. An increased incidence of leukaemia has been reported amongst radiologists as compared with other physicians (March, 1950), and a similar increased incidence of leukaemia in

doses in short periods. This probably accounts for the short latent periods of the cases reported by Hesse in 1911.

TABLE II

LATENT PERIOD FOR THE DEVELOPMENT OF SKIN CANCERS

No. of cases	Latent period in years		References
	Range	Mean	
Patients exposed to single or repeated courses of radiotherapy			
33	3–30	12	Holthusen and Englmann (1931)
26	7–26	17	Grutzmacher (1942)
20	4–22	14	Lacassagne (1945a)
11	12–48	32	Smithers (personal communication)
People exposed occupationally to chronic irradiation			
37	4–14	9	Hesse (1911)
6	19–34	26	Lacassagne (1945a)
15	7–35	22	Spear (personal communication)

For skin sarcomas Jones (1953) reported latent periods of 3–30 years, contrasting with the report of Walter (1950), who in three cases, following skin doses of 3,500–4,600r. stated that the first nodule appeared in 5, 11 and 14 weeks respectively. In two of these cases the histological report at 7 and 11 weeks respectively, was grade 1 carcinoma, while in the third case a skin papilloma was diagnosed after 19 weeks. If these are true cases of x-ray cancer, and not instances of "pseudo-epitheliomatous hyperplasia" (Grinspan and Abulafih, 1955) similar to those associated with some infectious processes (for instance leishmaniasis, blastomycosis, tuberculosis, syphilis) then the latent periods involved are much shorter than have previously been reported.

Skin lesions antecedent to the appearance of cancer

The suggestion of Lacassagne (1945a) that continuous irradiation incurred as an occupational hazard is associated with a longer latent period than short concentrated courses of radiation given in treatment, though unsupported by evidence at the moment, may still prove to be correct. The skin lesions developing from these two types of exposure have a *different history*. The chronic radiation dermatitis of radiologists is characterized by the slow development of keratoses and warts together with vascular damage in the dermis leading to extreme vulnerability of the skin and to indolent ulceration. The acute radiation dermatitis induced in radiotherapeutic procedures starts with an erythema, may develop into moist desquamation, heal and later result in an atrophic, irregularly pigmented scar, with atrophic changes and telangiectasis. This scar also ulcerates and the edges may become malignant. The vascular changes, hyalinization of stroma leading to contracture of the scar, the tendency to ulcerate and the abnormalities in pigmentation are, however, found in the late stages of both acute and chronic radiodermatitis. According to Hesse (1911) the edges of the ulcerations in the

Amongst the x-ray cancers the basal-cell variety is more frequent in younger patients: the average age of 5 patients with basal-cell cancer being 19 years, as against 32 years for 35 patients with squamous-cell cancer; of 6 patients under the age of 20 years, 4 had squamous-cell cancer and 2 basal-cell cancer, as against 92 and 6 for the whole series. As well as age, the site of the lesion may affect the type of cancer developing after irradiation. Of the spontaneous skin cancers in the white races 80 per cent or more are localized in the face and head region and the majority of tumours in this site are of the basal type.

The occupational x-ray cancers of the skin are localized largely on the hands, arms and chest, which are the regions in which squamous-cell carcinoma is more common in the spontaneous group. Anderson and Anderson (1951) reported 10 cases of basal-cell cancer in the face following radiotherapy and attributed the presence of basal-cell rather than squamous-cell carcinoma to the general predominance of basal-cell cancers at these sites.

Sarcomas of the skin have been reported following irradiation of normal skin, and especially after the treatment of lupus (Coenen, 1909, Deuticke, 1939). A critical review of radiation sarcomas of the skin has been published by Jones (1953). In addition to 2 cases of sarcoma in persons exposed occupationally to radiation, he listed 8 cases of skin sarcoma following radiotherapy for psoriasis, hypertrichosis, endothelioma of the dura, Graves's disease, keloid and papilloma of the bladder. An additional 4 cases have been recorded by Pettit, Chamness and Ackerman (1954). However, the total number of reported cases is small and skin sarcoma is probably a rare sequel to radiation injury.

Predisposing conditions

The treatment by radiation of benign dermatoses has occasionally resulted in malignant change, as mentioned in the case of lupus. Some of these skin lesions predispose to tumour formation and lupus is a particularly well-known example, but psoriasis and some forms of eczema can also become cancerous without radiation treatment. As the frequency of such changes is not known it is impossible to estimate how much the incidence of tumour formation in these lesions is increased by radiation. Lacassagne suggested that a shorter latent period for the appearance of lupus carcinoma in the irradiated patients may indicate the carcinogenic action of radiation in this condition. In 13 irradiated patients the latent period was 25 ± 2.7 years from the onset of lupus as compared with 38 ± 5.0 years for 6 patients not treated by radiation.

Latent period

Lacassagne (1945a) suggested that persons chronically exposed to radiation in their occupation develop skin cancers later (that is 26 years on an average) than do patients exposed to single or repeated courses of radiotherapy where he reported an average of 13-14 years.

In Table II, however, the data are collected of various workers who quoted latent periods for skin cancer development in patients exposed to concentrated courses of radiotherapy and in chronically exposed radiological workers. No significant difference is apparent, which is not surprising since, particularly in the early years of radiology, the occupationally exposed persons may have received considerable

Cilley, Kirklin and Leddy (1935) and Cilley, Leddy and Kirklin (1935a and b) found that, on the basis of ionization measurements, the average dose to the palm of the hand received by a radiologist in a well-run diagnostic department where no protective clothing except leather gloves was worn was about 0.9 s.e.d.* (300–400r) per year, for a period of 8 years. The dose to the finger tips was about three times as great The authors reported no radiation damage. If the dosage figures can be trusted two conclusions are drawn, namely, that a dose of the order of 1,000r per year to the finger-tips for 8 years produces no appreciable damage, and, if 1,000r per year is received by the finger-tips in a well-run department, the yearly dose may have been much greater than this under less well-organized conditions.

It is concluded, therefore, that some of the early radiodiagnosticians may have received skin doses of the order of tens of thousands of roentgens during their working career, and the very severe skin lesions preceding the appearance of malignancy is consonant with such a dose. Also relevant is the case reported by Hultberg and Larsson (1950) of a dentist who had received to each hand a dose estimated at 15,000r over a period of five years, due to holding films in patients' mouths. The hands showed erythema, desquamation and blistering, but after he ceased handling films, the reaction subsided. The inference is that a dose of 15,000r in five years was less than that received by the early radiologists who showed such severe and permanent skin damage.

Histogenesis of skin cancer

The most constant histological features of chronic radiation dermatitis and of late stages in acute dermatitis are the vascular changes in the form of endarteritis obliterans, periphlebitis and telangiectasis. These changes vary considerably over the lesion and even in the same area normal and injured arterioles may be found. The hyalinization of fibre bundles of the derma with some breakdown in the form of elastosis, oedema and infiltration, disappearance and clumping of elastic fibres are the consequences of non-specific inflammatory processes and of the vascular damage leading to ischaemia (Teloh, Mason and Wheelock, 1950). The epidermal changes also vary with size of field, and foci of hyperkeratosis and acanthosis may alternate with regions of epidermal atrophy and loss of rete pegs. Hair follicles are usually absent, the arrectores pilorum may degenerate or swell and the sudoriferous glands be grossly distorted Teloh, Mason and Wheelock (1950) analysed 215 lesions of radiation dermatitis and found 51 invasive and 4 pre-invasive carcinomas. Most of the carcinomas were in the lowest grade of malignancy. In addition 47 lesions showed "dysplasia," that is hyperchromatosis of cells in the deeper layers, clumping of chromatin, abnormal size of nuclei and nucleoli, presence of abnormal mitosis and disproportion in volume of nucleoli, nuclei and cytoplasm. These changes are considered by the authors to be pre-cancerous and more sinister than the premature or unequal keratinization found in a number of lesions.

Bone tumours

Relative to the radiation conditions bone tumours are the best documented class of radiation-induced malignancies in man, but the quantitative picture is

* s e d = skin erythema dose.

chronic dermatitis of radiologists are also the predominant regions where cancers develop, though some may occur in the keratoses.

It is not known what degree of skin injury is necessary to initiate carcinogenesis. In the reports by Hesse (1911), Holthusen and Englmann (1931), Grützmacher (1942) and Lacassagne (1945a and b) there was excessive skin damage over a fairly wide region in which foci of malignancy appeared but, on the other hand, without these changes a tumour would probably not have been ascribed to radiation. It cannot be proved from the clinical data that this severe skin injury is necessary for radiation carcinogenesis. If Walter's cases are accepted as definite carcinomas it appears that a moderate erythema is sufficient for subsequent and rapid tumour production. In all cases of cancer following minor radiation damage to the skin the causal relationship must remain uncertain. It is feasible that minor radiation injury may merely determine the localization of an existing tendency to malignancy. The promoting action of sunlight in the development of skin tumours in xeroderma pigmentosum may be a parallel example of a promoting and localizing action.

Various workers have given data on the incidence of malignant change in chronic radiation dermatitis and the estimates range from 10 per cent to 28 per cent, as seen from Table III.

TABLE III

INCIDENCE OF MALIGNANT CHANGE IN PATIENTS WITH SEVERE
RADIO-DERMATITIS

No. of patients	No. with cancers	Percentage	References
259	27	10	
97	20	21	
80	20	25	
171	34	28	

Where cancers occur they are often multiple. Teloh, Mason and Wheelock (1950) found 20 patients with single and 14 with 2–4 cancers and Hesse (1911) reported multiple tumours in 17 of 54 cases.

The incidence of severe radiodermatitis following radical x-ray therapy is unknown. Mild degrees of radiodermatitis were estimated by Sulzberger, Baer and Barota (1952) to occur in about 25 per cent of patients after radical x-ray treatment for malignant skin conditions No evidence was found for persistent skin changes in patients with benign dermatoses treated with fractionated doses up to 1,400r.

Radiation dosage and production of skin cancer

In view of the close association between radiation-induced skin cancer and severe radiation dermatitis in exposed workers, it would be of interest to know the dose they received but this reconstruction is a formidable problem and except in special cases may be impossible.

Wintz and Rump (1931) stated that some early workers received doses of at least several roentgens per day to the hands, so that skin doses of 1,000r or more per year were received.

one with a terminal body burden of about 50 micrograms of radium element, and the other with about 8 micrograms of radium element. The autoradiographs showed the radium to be limited to about 10 per cent of the Haversian systems in the section from one patient and 2 per cent in the second patient. Within the Haversian systems the radium was apparently uniformly distributed. Because of its very limited distribution the α-ray dosage from the radium in the Haversian systems could reach high values. Hoecker and Roofe calculated dosages of about 10 rep per day, so that in one year a dose of thousands of rep would be delivered to certain areas. Recently Spiers (1953) has made more precise calculations of the radiation dose, using the data of Hoecker and Roofe, obtaining similar values.

There is likely to be substantial excretion of radium during the lifetime of the patient (Norris, Speckman and Gustafson, 1955) so that radiation doses calculated on the basis of the terminal body burden will be underestimated.

Clinical course

The review of Looney (1955, 1956) included the dial painters and a group of 50 persons given radium salts for therapeutic purposes in which 5 cases of bone tumours were reported. In these 5 cases the terminal radium burden varied from 0.8 to 8 micrograms. Looney reviewed the skeletal radiographic changes observed in these groups. Increased density in cancellous bone and well-defined areas of decreased density in compact bone, as well as changes in normal configuration were observed. For terminal body burdens below 0.5 micrograms of radium almost no radiographic changes could be seen. Above 0.5 micrograms of radium, major skeletal changes were sometimes observed but these are determined not only by the radium burden. Some patients with a burden of 0.5-1 microgram of radium showed major skeletal changes and tumour formation was sometimes seen, whereas in other patients with 10-15 micrograms the changes were of a minor nature. Looney stated that "this marked variation in clinical response emphasizes the importance of the dynamic relation between the destructive and reparative processes of the body in the eventual production of clinical change."

As regards the development of malignancy, Looney reported, "there is a latent period followed by symptoms which usually develop from changes in weight bearing bones and bones which are subject to repeated trauma. Later tumour formation occurs at a site which is usually not that of the initial symptoms. When malignancy occurs there may be a marked accentuation of the disease process and death usually occurs about 1 to 4 years after the initial symptoms at the site the tumour develops."

Histopathology

According to Looney the most striking histopathological changes are: (1) The formation of atypical osseous tissue, laid down around trabeculae of cancellous bone, and the filling of the intertrabecular spaces by an acellular fibrous tissue; and (2) areas of destruction in compact bone with replacement of bone by fibrous tissue. Malignancies develop in or around areas of proliferating fibrous tissue and atypical osseous tissue. These areas are found particularly at the end of the bones, which are the preferential sites for the formation of the atypical bone tissue and not in the regions which are more commonly affected by aseptic bone

incomplete. The main sources of data are the reports of bone tumours in the radium dial painters, in patients who have received injections of radium salts for therapeutic purposes and in patients following treatment involving irradiation of bone.

Incidence of bone tumours following ingestion and injection of radium salts

The data on the radium dial painters is unique in that the total population at risk is known. From Martland's study (Martland, Conlon and Knef, 1925, Martland, 1931) it is known that between 1917 and 1924 a total of 800 girls, but not more than 250 at a time, were employed as dial luminizers in a New Jersey factory. The paint used was crystalline phosphorescent zinc sulphide in gum acacia rendered luminous by the addition of small amounts of radium, mesothorium and radiothorium present as insoluble sulphates. The paint was applied to the watches by a brush which the luminizers pointed with their lips. Each luminizer painted 250-300 watches per day and licked the brush up to 14 times per dial. Martland estimated that in six months up to 5 milligrams of radio-active element could be swallowed. He reported in 1925 the findings of anaemia and jaw necrosis in some of the girls but there was no mention of malignancy. In 1931 Martland reported that between 1922 and 1928 there were 13 deaths from anaemia and jaw necrosis, occurring 4-6 years after leaving employment and 5 cases of osteogenic sarcomas.

Eight more cases of malignancy among dial painters apparently attributable to radium ingestion were reported by Aub and his colleagues (1952) and Looney (1955, 1956). In addition five cases of bone tumour were found among patients treated with radium by mouth and intravenously for various disorders, including hypertension, arthritis and anaemia, during 1915-1930.

Radiation dosage

It was possible to make measurements of the body burden of radioactive element in the case of some of the dial painters and radium injected patients who had died as a result of the radiation effects. This is summarized and discussed by Aub and his colleagues (1952) and Looney (1955, 1956). The terminal body burden of radium in those patients who developed osteogenic sarcomas varied from 0.5 to 50 micrograms. This may appear a small amount of radioactive material to produce such severe changes, but several points must be considered. First, the early measurements were made only of radium, and other radioactive elements of shorter half-life, such as mesothorium, might have contributed largely to the initial radiation damage, though not to the final measurement of the activity. Secondly, the radiation dose received by a given part of the bone will depend on the concentration of radium at that point, and a very non-uniform distribution of the radioactive material might lead to high levels of radiation dosage in certain areas.

With regard to the first point, the possible role of mesothorium has been investigated by Evans (1950), who suggested that the radiation dose might, in certain cases, be increased several times above that calculated for radium alone.

In connexion with the non-uniformity of the radium distribution in the bone valuable autoradiographic studies were made by Hoecker and Roofe (1951), using bone sections from two of the dial painters who had died with osteogenic sarcoma,

and an osteosarcoma in the skull 7 years after massive radiotherapy for a retino-blastoma.

The other cases of Cahan and his colleagues (1948) followed the treatment of benign bone lesions. It cannot be decided whether radiotherapy initiated the malignant process or merely accelerated the development of malignancy inherent in the benign conditions. The role of tuberculosis as a predisposing condition is emphasized by the fact that 13 cases reported in the literature (and reviewed by Cahan and his colleagues, Jones, and Lacassagne) occurred after the treatment of this condition and that 10 of them were localized in the knee joint. Jones reported a case of an osteosarcoma of the jaw following radiotherapy for actinomycosis. Altogether 39 cases of bone tumours arising after radiotherapy have been collected, 8 occurring in normal bones and the remainder in benign bone lesions. For 38 of these cases Jones found an average latent period of 8·6 years with a range of 3 to 22 years.

In the cases of radiation-induced bone sarcomas reviewed by Jones, the dose reported given was 3,000–4,000r or more. As already mentioned, however, a determination of the radiation dose absorbed in bone is not straightforward. Depending on the quality of the radiation and on the position within the bone at which the dose is delivered the radiation energy absorbed may be considerably greater, or in some circumstances less, than that in soft tissue for the same dose in roentgens (Spiers, 1949, 1951).

To summarize, it appears from the reports that a bone tumour following radiation treatment is rare; only two case reports of osteogenic sarcoma in the ribs after irradiation following mastectomy have been found in spite of the large number of these treatments that have been given. However, since the population at risk is not known, no estimate of actual frequency can be made.

Regarding the bone tumours produced by external radiation there is little data on the histological processes preceding the production of the malignancy and, in particular, on the degree of radiation osteitis present. In the radium cases, however, bone changes were shown by all patients who subsequently developed tumours. From Looney's data on these cases it appears that bone tumours were observed in about one-fifth of the cases showing marked bone changes.

Leukaemia

During the last forty years reports have appeared suggesting that leukaemia incidence may be particularly high among radiation workers (Furth and Lorenz, 1954). No figures relating to actual increase in incidence could, however, be given without knowledge of the population at risk, and this was first attempted by March (1944, 1950), who based the values for the incidence of leukaemia in radiologists and in non-radiological physicians over two periods 1929–44 and 1944–48, on obituaries in the *Journal of the American Medical Association*. His figures are set out in Table IV.

The difference in incidence, a factor of 9, is very considerable, but two considerations must be borne in mind. Firstly, there are large differences in leukaemia incidence between various social classes in the population for which there is at present no explanation (Henshaw and Hawkins, 1944), and, secondly, the completeness of March's data is doubtful. The difficulty of making any reliable estimate of the doses received by the early radiologists has been discussed; a

necrosis. Relatively little or no radioactivity was detected by means of autoradiography in and around the atypical osseous tissue. Looney considered that the deposited radio-elements initiate a sequence of events leading to pathological alterations which are probably the end-result of many augmenting factors such as trauma, damage to the blood supply, decreased bone repair and increased bone destruction. He stressed the lack of correlation between the localization of radio-elements and of radiographic, pathological and histological changes.

Latent period

The latent period between the time of deposition and the development of symptoms of malignancy appears to decrease with increased burden of radium. In the first group of dial painters, analysed in 1931, the mean latent period was 9 years. In the second group of dial painters and in the group of patients given radium therapeutically, analysed in 1951, the average body burden was about one-eighth of that in the first group, and the mean latent period was about 25 years. There seems, however, in this series to be a definite threshold level for the development of malignancies at about 0.5–1 microgram of radium as terminal body burden, and there can be no justification for assuming a linear relationship between dose and production of malignancy. The average age of the dial painters was 17 years at the time they started work with radium, whereas the average age of the treated group was 39 years. The results give no indication that age at the time of deposition is a major factor in the production of the radiation effects.

Bone tumours following radiation treatment

Bone tumours have also been reported following the radiation treatment of benign bone lesions, of osteomyelitis, of bone and joint tuberculosis, of chronic arthritis and very rarely in normal bone following treatment of overlying benign or malignant lesions. Reviews and additional cases have been published by Lacassagne (1945a and b), Hatcher (1945), Cahan and his colleagues (1948) and Jones (1953) The most convincing cases are those where osteosarcomas followed the irradiation of normal bones in the treatment of other conditions. This group comprises the cases of Hatcher, where a tumour in the fibula and one in the ulna were observed 4 and 11 years after the treatment for tumours of the tibia and of the radius respectively, and in a third case a chondrosarcoma in the seventh rib followed 12 years after post-operative irradiation after radical mastectomy. Spitz and Higginbotham (1951) observed a bone sarcoma in the lumbar spine 4 years after prophylactic irradiation (dose of about 5,000r) of the pelvic node region following orchidectomy for seminoma. A similar case of osteosarcoma following a dose of 4,000r of 200 kV x-rays given in 70 days after orchidectomy was reported by Auerbach and colleagues (1951). In this instance the tumour probably arose in the paravertebral tissue and invaded the bone secondarily. Jones (1953) quoted a case of Kaae and Glann in which a fibrosarcoma of the mandible was found 13 years after a high radium dosage for a gingival epithelioma, and 2 cases of Wolfe and Platt where a tumour in the nasal bones followed radiotherapy for dermatoses. Cahan and his colleagues (1948) reported an osteosarcoma of the second rib 11 years after post-operative irradiation following radical mastectomy

interest centres around the experiences of the uranium miners of Schneeberg and Joachimsthal.

Lung cancer in uranium miners

Since the sixteenth century there have been reports of a specific miners' sickness at Schneeberg (now in Eastern Germany) and at Joachimsthal (now in Czechoslovakia), but not until the end of the last century at Schneeberg and later at Joachimsthal was it recognized that many of the miners were dying of malignant disease, at first erroneously diagnosed as lymphosarcoma, and later correctly diagnosed as lung cancer. A great deal of dust was present in these mines possibly containing traces of arsenic, cobalt and other metals and the high incidence of lung cancer was first ascribed entirely to these materials. However, when a high level of radioactivity in the air of these mines was observed this was considered a possible cause of the disease. A number of papers and reviews have appeared on this subject, those by Lorenz (1944) and Šikl (1950) being of particular interest.

Figures given by Peller (1939) for the Schneeberg miners, together with comparative figures for males in Vienna are shown in Table V.

TABLE V

CRUDE CANCER MORTALITY PER 1,000

| | Miners in Schneeberg | | Vienna. Males 15–79 years of age |
	1875–1894	1895–1912	1932–36
Lung cancer	12 7±1 0	16 5±1 5	0 34
Cancer of other organs	2 4±0 4	2 1±0 6	2 1

At Joachimsthal lung cancer was not recognized as a major cause of death among the miners until just before 1930, but the findings reported by Šikl (1950) for the periods 1929–30 and 1933–38, show clearly the prevalence of cancer of the lung in miners dying at that time. Of the 52 deaths occurring over the second period about half were due to cancer of the lung and about half due to silicosis or tuberculosis, or a combination of both, as shown in Table VI, taken from Šikl (1950)

TABLE VI

CAUSE OF DEATH OF JOACHIMSTHAL MINERS 1933–38

Age class	Cancer of lung	Silicosis tuberculosis	Other causes*
20–39	2	10	3
40–49	11	12	2
50 and over	7	1	—
Total	20	23	5
Average age at death	47 6	40 8	

* Excluding 4 violent deaths.

TABLE IV

Period	Non-radiological physicians		Radiological physicians	
	No. of deaths	Leukaemia	No. of deaths	Leukaemia
1929–44	50,285	221 (0·44%)	175	8 (4·57%)
1944–48	15,637	113 (0·72%)	124	6 (4·84%)
Total 1929–48	65,922	334 (0·51%)	299	14 (4·68%)

number of them must have been exposed to several hundred roentgens per year. However, the radiation received by these workers was relatively soft, and the absorption by clothes,.together with other factors, would lead to a very non-uniform dose on and in the body, and the significance of such non-uniformity of dose on leukaemogenesis in man is unknown.

The second large group of data relating to radiation-induced leukaemia in man is provided by the atomic bombing of Hiroshima and Nagasaki. There are various reports including that of Lange, Moloney and Yamawaki (1954), indicating that the incidence of leukaemia (mainly of the myeloid type) has been much greater among survivors within two to three kilometres of the centre of the explosion than among those at a greater distance. Too great a reliance cannot be placed on the actual values of incidence quoted since the follow-up of survivors is still proceeding and attempts are being made to sort out the population movements that will affect the statistics. The radiation dose received by any given survivor is, and will remain, very uncertain, but the whole-body dose cannot exceed a few hundred roentgens, since the mean lethal dose for man is believed to be about 400 roentgens. It is likely that in many cases the dosage was very non-uniform.

The third group of human data which may have a bearing on the radiation induction of leukaemia concerns persons with ankylosing spondylitis treated with x-rays (Court Brown and Abbatt, 1955). The preliminary report on 9,364 patients treated from 1940 to 1954 showed an incidence of leukaemia at least five times and possibly ten times the expected number for a general population. Among those patients given more than one course of treatment the observed deaths are probably at least nine times those expected to occur. It is possible that the bone marrow of patients with ankylosing spondylitis is abnormal and particularly susceptible to radiation and to the development of leukaemia after exposure.

The range of treatment techniques and doses given in this series is very great, and more data are required before definite conclusions can be reached.

Lung tumours

Incidence

No reports have been found in the literature of lung cancer following radiation damage to the lung by x-rays or γ-rays. There have been at least two reports (Abrahamson, O'Connor and Abrahamson, 1950, and Vogtlin and Minder, 1952) of lung cancer following the injection of Thorotrast used as a radiographic contrast agent, but the evidence of Thorotrast being the causal factor is not convincing in either case. Regarding a possible connexion between lung cancer and irradiation

to assess the role of radiation in the production of lung cancer in these miners

Apart from the miners at Schneeberg and Joachimsthal there have been no other reports of an abnormally high incidence of lung cancer in conditions where a high radon content of the atmosphere might be expected; this was discussed by Lorenz (1944). However, such a negative result must be treated with great reserve. It is rare to meet conditions where any quantitative assessment of risk can be made, as was the case in the relatively closed communities of Schneeberg and Joachimsthal.

Radiation-induced tumours at other sites

With the exception of tumours in the neck region and sarcomas of the tongue, reports of radiation-induced cancers at other sites are scanty or unconvincing.

Female genital tract

In the female genital tract the incidence of ovarian tumours following artificial menopause induced by radiation varies in different series between 0.2 and 1 per cent and is no greater than might be expected for the general population over the age of 40 years (Speert, 1952). Furthermore, this treatment is given to patients with some disorder of ovarian function which itself may predispose to tumour formation. Speert reported that in different series only 2–3.5 per cent of patients with ovarian cancers had previous pelvic irradiation. Though 8 per cent of patients with cancer of the corpus had previous irradiation to the pelvis, on the average about 8 years previously, this might be attributed to a slowly developing lesion requiring the radiation treatment and possibly a speeding up of the malignant process.

Liver

In the liver a haemangioendothelioma has been reported by Ross (1932) and attributed to the effect of radiation from a stray radium needle embedded in the interventricular septum of the heart. An endothelial-cell sarcoma following an intravenous Thorotrast injection has been reported by McMahon, Murphy, and Bates (1947).

Breast

Lacassagne (1945a) stated there are no reliable reports of breast cancer due to radiation.

Tongue

There are at least 6 cases reported of sarcoma following the treatment of squamous-cell carcinomas or of a papilloma of the tongue by interstitial radium insertions. Of these cases 4 have been reported by Gricouroff (1937, 1943), 1 by Deller (1951) and 1 by Marquès, Planel and Hermons (1952). The average latent period varied from 4 to 19 years with an average of 9 years.

Neck and thyroid

Tumours in the neck region have been reported by Goolden (1951) and by Raven and Levison (1954), who collected 11 cases in the pharynx and 1 case in

The population of miners over this period was about 400, and the annual mortality rate for lung cancer works out at about 10 per 1,000, comparable to the values for the Schneeberg miners given above.

Over the period 1933–38 the histological types of lung cancer found by Šikl were: epidermoid 9, oat-cell 10, polymorph sarcoma 1. Metastases were found in all cases.

Data regarding the length of time spent in the mines before the development of the disease are only available for 9 lung cancer cases of Šikl between 1929 and 1930. In these 9 cases the average duration of work was 17 years and the shortest time 13 years

Radioactivity of the mines and radiation dosage to the lungs.—Evans and Goodman (1940) gave a summary of the various measurements made of the radon concentration in the air of the various mines at Schneeberg and Joachimsthal. There is a range of variation of more than ten to one in the concentrations measured within the same mine and a variation of up to eight to one in the mean values for the different mines. The average value for all mines is about 3×10^{-9} curies per litre, and this value is taken as the basis for an estimate of the radiation dose to the lung, though it must be remembered that the ventilation of the mines had been improved by the time the radon measurements were made, and that previous values of radon concentration may have been much higher than those measured.

The radiation dose to the lungs produced by breathing a radon-rich atmosphere is not, however, mainly due to the radon itself, but to the short-lived solid daughter products also carried into the lung. For this reason the dust content of the atmosphere is of great significance, affecting the quantity of daughter products inhaled and, also, since dust will deposit selectively in the respiratory tract, leading possibly to relatively high local dosage in certain regions.

Calculations of the dose received by the tracheal epithelium have been reported by Bale and Shapiro (1955). For normal dusty air and a concentration of 3×10^{-9} curies radon per litre the mean dose is about 0.2 rad per day. In 10 years the cumulative dose would be about 600 rad. Owing to selective deposition the dose might be more than 10 times the calculated average dose, so that in a lifetime the uranium miners might have received a dose to certain parts of the lung of 10,000 rad of α-rays.

It cannot be assumed, however, that radiation is necessarily the whole cause of the incidence of lung cancer in the miners. As stated, a great deal of dust was present in the air of mines, containing traces of arsenic, cobalt and other metals which might have a carcinogenic or a co-carcinogenic action. The incidence of silicosis and tuberculosis in the Joachimsthal miners indicate the serious effects of the dust, and a possible causal connexion between these lung conditions and cancer must be considered Šikl pointed out, however, that on the whole silicosis, though present, was not extensive in the cases of cancer of the lung The lungs most heavily affected with silicofibrosis were generally free from malignant growth It is to be noted, from Table VI, that the average age of death from silicosis and tuberculosis was slightly less than that for cancer of the lung. Silicosis and tuberculosis may have enhanced the carcinogenic effect of radiation, as tuberculosis does in other sites, and silica in the animal experiments of Burrows, Mayneord and Roberts (1937). These factors, in addition to the frequency of respiratory troubles due to poor working and living conditions (see Lorenz, 1944) make it difficult

thyroid arising in adolescents following irradiation in infancy. In the latter case interference with endocrine balance may play a part.

The role of tuberculosis and some other inflammatory conditions in enhancing the carcinogenic effect of radiations appears established for tumours of the skin and bone. The periphery of areas of chronic radiation damage appears to be the site of origin of radiation-induced tumours of the skin, and this may also apply to the bone tumours.

Little is known about the processes intervening between the irradiation and the subsequent malignant change, and whether severe injury with incomplete regeneration is a constant and necessary condition for later malignant change.

As late radiation effects are as a rule available only for human studies and data on radiation doses are incomplete, even if available, animal experiments must be used to solve many of the outstanding problems, *which can be summarized as* follows: (1) Variation of tumour incidence with (*a*) dose of radiation, (*b*) dosage rate, protraction and fractionation of dose, (*c*) volume of tissue irradiated. (2) The susceptibility of tissues and organs to the carcinogenic effects of radiation and its variation with (*a*) individual sensitivity and sex, (*b*) the functional activity of the tissue and the influence on it of hormones, (*c*) the presence of predisposing factors such as tuberculosis and other inflammatory conditions. (3) The mechanism of action of radiation as a carcinogen; this involves an enquiry into the processes intervening between the actual irradiation and the onset of malignancy, and a comparison of these processes with those in the development of cancers induced by chemical carcinogens.

The animal experiments which are summarized are chosen only for their value *in elucidating the problems* resulting from the analysis of the human data and are not intended as a complete catalogue of the experimental work done.

EXPERIMENTAL INDUCTION OF CANCER BY RADIATION

Introduction

The first successful attempt to demonstrate the carcinogenic effect of radiation in animals was reported in 1910 by Marie, Clunet and Raulot-Lapointe. They obtained a sarcoma in a rat treated over the sacro-ischial region by repeated small doses of radiation which accumulated to about 13,000r in 18 weeks (Lacassagne, 1945a). Bloch (1924) was the first to produce a carcinoma, by localized irradiation of a rabbit's ear, with a dose amounting to about 38,000r in 32 months. This result was confirmed by Schürch (1931a and b).

Carcinogenesis by localized radiation was studied also by Goebel and Gérard (1925), using guinea-pigs, Jonkhoff (1928) and Sedginidse (1933), using mice, and Ludin (1934), who produced a sarcoma in the knee joint of rabbits. In all these experiments fractionated small doses were given over a long period of time and tumour formation occurred after a protracted interval following a large total dose, thus reproducing the conditions under which skin and bone tumours developed in radiological pioneers and patients.

The first report of a single dose of x-rays, of 1,000r only, acting as a carcinogenic agent is due to Lacassagne and Vincent (1929), who produced sarcomas in rabbits on irradiation of an abscess caused by *Streptobacillus caviae*. Later work of Lacassagne (1933) and of Burrows, Mayneord and Roberts (1937) showed that

the thyroid gland. In 7 of these cases intensive x-ray treatment was given for thyrotoxicosis and in the remaining 5 for tuberculous cervical lymphadenitis. In all but one of Goolden's cases there was evidence of severe skin injury. The latent period varied from 13 to 30 years and averaged 24 years. The thyroid tumour was an adenocarcinoma, and of the 11 tumours of the pharynx region 10 were carcinomas and one a sarcoma.

An increased incidence of thyroid tumours has been reported recently following x-radiation of the neck region in infants for thymic enlargement and other neck conditions. Duffy and Fitzgerald (1950) reported that 10 of 28 patients with cancer of the thyroid under the age of 18 had previous radiation of the thymus. Simpson, Hempelmann and Fuller (1955) surveyed 1,722 children treated by x-rays for thymic enlargement and compared them with 1,795 children not treated. Of the first group only 1,400 children were traced and in this group 7 patients with leukaemia, 6 with thyroid cancer and 9 with thyroid adenomas were found as against 1 with a thyroid adenoma and none with leukaemia or thyroid cancer in the control series. The treatment doses varied from 50r to 1,500r with the majority below 600r. In 604 of the cases which received less than 200r 3 patients with leukaemia but none with thyroid tumours were found, while in 804 cases treated with more than 200r there were 4 patients with leukaemia, 6 with thyroid adenomas and 6 with carcinoma of the thyroid. Clark (1955) reported at the Geneva Conference 13 cases of thyroid cancer in adolescents under 15 years of age who had been irradiated over the neck region at ages varying from 2 months to 6 years. The treatment doses varied from 200r to 725r and thyroid cancers were found 3–10 years later. He also suggested that the increased incidence of thyroid cancers in children reported by Winship (1951) may be due to the greater use of radiation in treating thymic enlargement in them. The doses of radiation involved in these cases are surprisingly small and Hempelmann suggested that their carcinogenic effect may be due to interference with the inter-relationships of thymus and thyroid and of the pituitary in so far as the production of thyroid-stimulating hormone is concerned. These tumours may, therefore, be comparable with some of the endocrine tumours found after small doses of radiation in animals.

Summary

A number of cases of human cancer ascribed to previous irradiation have been described. This number is very small in comparison with the total number of persons who have been exposed to radiation either occupationally, or during treatment or investigation. Except for the radium dial painters, it is impossible to estimate the total population at risk, and in general, it is impossible to determine the frequency of malignant change following a given type of radiation exposure. The latent period for the development of radiation-induced tumours is very variable, but is frequently 10–20 years.

In skin and bone tumours, the dose of radiation received appears in the majority of cases to be of the order of thousands of roentgens and this applies to both concentrated and to chronic exposures. The human data are not adequate to determine the relative effectiveness of a given dose of radiation given over various periods of time.

The evidence suggests that smaller doses of radiation may be sufficient for leukaemia production (at least in concentrated exposure) and for tumours in the

colleagues (1953) had 32 Holtzmann female rats surviving an x-ray dose of 700r when kept in parabiosis for 30 days. They reported 1 basal-cell carcinoma and 1 fibrosarcoma in the region of one of the mammary glands.

Whole-body exposure to single doses of x-rays in male rats (Koletsky and Gustafson, 1955) produces fibromas of the skin in either the dermis or the sub-cutaneous region. Of 123 rats surviving a dose of 660r 40 had a total of 118 such tumours, which often became very large.

In these experiments the incidence of tumours of the skin is relatively small and varies between 5 and 16 per cent. None of these experiments give any information about the mechanism of carcinogenesis, but establish that "small" doses given as whole-body radiation may play a role in the formation of skin tumours. Whether doses of the lethal range should be described as "small" simply because very much greater dosages are tolerated when localized over parts of the body is another problem.

Though not strictly whole-body radiation it is convenient here to discuss the work with external β-sources irradiating the surface of the body. A great variety and number of skin tumours and of subcutaneous tumours has been reported by Henshaw, Riley and Stapleton (1947), Henshaw, Snider and Riley (1949) and Raper, Henshaw and Snider (1951) following exposure of mice and rats to single, repeated and chronic irradiation by β-rays from ^{32}P. Unfortunately little histology was done on this material and the tumours are classified as cutaneous when in the skin, or subcutaneous when the skin was mobile above it. There is a suggestion that the latter tumours are associated with the breast since their spontaneous rate is much higher in female than in male Sprague-Dawley rats, as shown in the data of Henshaw, Snider and Riley (1949).

Henshaw and his colleagues compared the effect on rats of repeated doses of β-radiation (from ^{32}P) given over the whole skin surface, at 0.5, 5.0 and 50 rep per day, with those of single exposures of 4,500, 6,500 and 8,500 rep. Observations were continued up to 19 months after starting, although by this time all the animals given single exposures were dead.

Repeated doses of 0.5 and 5.0 rep per day appeared not to affect materially the incidence of the skin tumours, but at 50 rep per day tumours began to arise about

TABLE VII

TUMOUR INCIDENCE FOLLOWING β-RADIATION

(data abstracted from Henshaw)

Group	Sex	No. of rats surviving	No. of rats with tumours	No. of tumours	
				Sub-cutaneous	Cutaneous
Controls at 19 months . ..	Male	51	8	8	1
	Female	37	24	33	1
50 rep/day for 19 months (≈ 24,600 rep)	Male	17	17	31	71
	Female	14	14	22	13
Single dose of 4,500 rep at 15 months	Male	11	11	20	73

a single small localized dose of irradition could cause a sarcoma in an inflamed region.

Tumour production following whole-body exposure to irradiation was first observed by Krebs, Rask-Nielsen and Wagner (1930), in experiments designed to reduce the immune response of mice to grafting. They reported the occurrence of lymphoid leukaemia. The first specially designed whole-body experiments to produce tumours were undertaken by Furth and Furth (1936) and Furth and Butterworth (1936) using single and repeated exposures. They found significant increases in the incidence of lymphoid and of myeloid leukaemia, of ovarian and lung tumours. Experiments with chronic whole-body irradiation on mice, rats, rabbits and guinea-pigs have been reported by Lorenz and his colleagues (1947) using β-rays on rats and mice.

Other series of experiments deal with tumour production following exposure to neutrons, to nuclear detonations and to the injection of various radio-isotopes. The early work has been summarized by Lacassagne (1945a and b), and later experimental work by Furth and Lorenz (1954), by Brues (1951, 1954), by Furth and Upton (1953), and by Furth and Tullis (1956).

Carcinogenic effect of whole-body radiation

For single whole-body exposure the dose necessary to produce malignant change in a reasonable proportion of the animals is generally found to be within the lethal range, so that many of the animals die before any tumours can appear. The main danger to survival in the acute syndrome is the depletion of the bone marrow and other blood-forming organs, and recently protection of the animals for the critical first few weeks has been achieved by establishing parabiosis between litter mates (Finerty and his colleagues, 1953), or by using various chemical "protective substances" injected prior to irradiation (Brecher, Cronkite and Peers, 1953). From these experiments and from those using sub-lethal doses (Koletsky and Gustafson, 1955) it is found that single radiation doses of from 600 to 1,000r induced in rats tumours at various sites in about one-third of the animals surviving for at least six months after exposure. The majority of these tumours occurred in the skin, kidney and bone, and are discussed under the various sites.

In the chronic experiments an effective dose of radiation is accumulated slowly, and consequently it is difficult if not impossible to determine the latent period. On the other hand, considerable doses of radiation are tolerated when applied in this fashion. It is also not possible to determine the dose necessary for tumour formation since in chronic experiments radiation is usually continued until tumours become visible or the animal dies, and thus a considerably greater amount of radiation may be accumulated than is actually required (Mole, 1955).

Skin tumours

Koletsky and Gustafson (1955) obtained 10 basal-cell carcinomas in 8 rats and 9 skin sarcomas in 9 rats in a group of 123 Wistar rats surviving for 6 or more months following a single dose of 660r of x-rays. Brecher, Cronkite and Peers (1953) had 21 female Sprague-Dawley rats surviving for 6 or more months after exposure to a single dose of 700–1,000r of x-rays. Among the tumours was a fibrosarcoma of the skin 11 months after exposure to 700r. Finerty and his

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Brown (1952) using C57 black mice, 30–36 days old, studied the influence of dose fractionation on the incidence of lymphomas at different dose levels. Table VIII gives some of their data for two dose levels. An optimal fractionation is found when the interval between treatments is 4–8 days. The existence of an optimal spacing suggests not only recovery from radiation effects but the balancing of various processes in the leukaemogenesis.

TABLE VIII

VARIATION OF LEUKAEMOGENIC EFFECT OF X-RAYS
WITH FRACTIONATION, ON C57 BLACK MICE

(*Kaplan and Brown*, 1952)

No of fractions	Overall time	Interval between fractions	Total dose			
			475r		673r	
			No. mice at risk	% lymphoma	No. mice at risk	% lymphoma
1	About 16 min.	——	29	41	—	—
2	1 day	1 day	26	35	14	64
4	3 days	1 day	27	33	44	66
8	7 days	1 day	27	37	25	52
4	12 days	4 days	51	76	32	75
4	24 days	8 days	49	61	43	93
4	48 days	16 days	50	24	50	70

At no dosage level did many leukaemias appear before 100 days, and at all dosage levels (except the lowest, 283r) practically all leukaemias had appeared by 300 days, though observations were continued until 600 days. With increasing dosage in the range of 283–475r single exposures raised the lymphoma incidence from 13 to 40 per cent. The total lymphoma incidence could, however, be increased to nearly 100 per cent by exposure to fractionated doses totalling 950r. Females were more susceptible than males and the initial number of mice per litter was also of significance. It is evident that a number of factors play a role in leukaemogenesis by irradiation

Further evidence for the complexity of the process is given by the work of Kaplan and his colleagues relating to partial body radiation (Kaplan and Brown, 1951). Shielding of part of one femur will greatly reduce the lymphoma incidence, as will whole-body irradiation to the upper and lower halves of the animal given with an interval greater than 24 hours. Related to these findings is the observation that intravenous injection of bone marrow suspensions made from the femoral marrow of 60-day-old mice can protect against the leukaemogenic action of whole-body radiation (Kaplan, Brown and Paull, 1953). This effect is maximal when the injection is made 1½ hours after irradiation and falls off when the interval between irradiation and injection is increased. The effect of protection is greater in males than in females. In mice given 673r in 4 fractions at 8-day intervals over 32 days (see Table VIII) injection of the suspension 1½ hours after exposure reduced the incidence of lymphoma to zero in 17 male and to 14 per cent in 14 female mice.

10 months after the start of treatment and all the animals had tumours by 19 months. Although the spontaneous incidence of tumours is higher in females than in males, the radiation-induced incidence was much higher in the males. This is evident in the 19 month results shown in Table VII which also includes certain figures for control rats, together with the tumour incidence at 15 months for male rats given a single dose of 4,500 rep.

A most important finding of these investigations was that single doses were much more efficient in the production of the skin tumours than the chronic exposures. It is seen in Table VII that at 15 months following a single dose of 4,500 rep the mean number of tumours per animal was greater than at 19 months following 50 rep per day for 6 days in the week, representing an accumulated dose of 24,600 rep. With single doses of 6,500 and 8,500 rep the number of tumours produced was considerably greater still. Henshaw and his colleagues mentioned that the generalized macroscopic skin changes before the appearance of skin tumours were much more severe in the rats given single exposures than those given 50 rep per day, and this may be an important factor in determining the relative effectiveness of concentrated and chronic exposure.

Bone tumours

Most experimental work on bone tumours has been carried out with local external radiation or, as discussed in detail later, by the use of radio-elements either introduced mechanically into the neighbourhood of bone or deposited metabolically after ingestion or injection. Koletsky and Gustafson (1955) have, however, reported finding osteogenic sarcomas in 5 out of 123 rats given whole-body radiation of 660r. The sites of the tumours were, humerus (2), tibia and fibula (2) and pelvis (1).

Thymic tumours and leukaemia

Whole-body radiation has been found to be a powerful leukaemogenic agent in mice, though in other species of experimental animal there are very few reports of radiation-induced leukaemia. Most of the experimental work has been with lymphomas originating in the thymus (Furth and Upton, 1953, Furth and Lorenz, 1954). Myeloid leukaemia appears to be a rare sequel of irradiation in mice.

In assessing the efficiency of radiation as a leukaemogenic agent in mice it is necessary to consider the influence of various factors on the incidence of these tumours. The spontaneous incidence varies with strain from almost 90 per cent in some to about 5 per cent in others. Within the same strain females may be more prone to the disease than males. The incidence of these tumours can be increased in some strains by oestrogenic hormones, orchidectomy, adrenalectomy, and by subcutaneous injection of carcinogenic hydrocarbons such as 9, 10-dimethyl-1, 2-benzanthracene. The incidence of lymphomas is reduced by thymectomy, androgenic hormones and cortisone (Gardner, 1953).

Single doses of whole-body irradiation as well as fractionated doses have been found to be leukaemogenic. Brues and his colleagues (1949), using CF₁ female mice, found that a whole-body irradiation of 400r of x-rays given over 10 days in doses of 40r daily was more effective in inducing lymphomas than a single dose of 400r, or the same dose given over 40 days in fractions of 10r daily. Kaplan and

Brown (1952) using C57 black mice, 30–36 days old, studied the influence of dose fractionation on the incidence of lymphomas at different dose levels. Table VIII gives some of their data for two dose levels. An optimal fractionation is found when the interval between treatments is 4–8 days. The existence of an optimal spacing suggests not only recovery from radiation effects but the balancing of various processes in the leukaemogenesis.

TABLE VIII

VARIATION OF LEUKAEMOGENIC EFFECT OF X-RAYS
WITH FRACTIONATION, ON C57 BLACK MICE

(Kaplan and Brown, 1952)

No of fractions	Overall time	Interval between fractions	Total dose			
			475r		673r	
			No. mice at risk	% lymphoma	No. mice at risk	% lymphoma
1	About 16 min.	—	29	41	—	—
2	1 day	1 day	26	35	14	64
4	3 days	1 day	27	33	44	66
8	7 days	1 day	27	37	25	52
4	12 days	4 days	51	76	32	75
4	24 days	8 days	49	61	43	93
4	48 days	16 days	50	24	50	70

At no dosage level did many leukaemias appear before 100 days, and at all dosage levels (except the lowest, 283r) practically all leukaemias had appeared by 300 days, though observations were continued until 600 days. With increasing dosage in the range of 283–475r single exposures raised the lymphoma incidence from 13 to 40 per cent. The total lymphoma incidence could, however, be increased to nearly 100 per cent by exposure to fractionated doses totalling 950r. Females were more susceptible than males and the initial number of mice per litter was also of significance. It is evident that a number of factors play a role in leukaemogenesis by irradiation.

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The reduction was less with a delay of 24 hours (Kaplan and Brown, 1951, Kaplan and his colleagues, 1955) and negligible after a delay of 4 days.

The role of the thymus in the development of lymphomas is of great interest. Thymectomy prevents or reduces the development of lymphomas in irradiated mice, but irradiation of the thymus is not necessary for the tumour development (Lorenz and Eschenbrenner, quoted by Furth and Lorenz, 1954) Kaplan, Brown and Paull (1953) and Kaplan and Brown (1954) grafted non-irradiated thymic glands from litter mates into the axilla of irradiated but thymectomized mice and obtained tumours which demonstrably started in the grafted non-irradiated thymus. On the other hand, in Kaplan's experiments, shielding of the thymus suppressed the incidence of lymphomas. Lacassagne (1954) pointed out that after grafting, as after irradiation, degeneration of thymic cells occurs before regeneration sets in and that the systemic changes induced by radiation stimulate the regenerating thymus to lymphoma production.

Lorenz and his colleagues (1947) used LAF$_1$ mice for their studies with chronic exposure to γ-rays at various daily dose rates. At daily exposures of 8·8r the incidence of lymphomas was significantly increased and the latent period shortened as compared with controls. At levels of 4·4r per day and below, the incidence of lymphomas was not increased, though there is a suggestion that at this dosage rate the latent period was decreased.

In rats, Koletsky and Gustafson (1955) found only 2 lymphosarcomas among their 123 rats surviving 660r of whole-body x-irradiation for 6 months or more. Furth and Lorenz (1954) quoted Metcalf and Inda who observed some leukaemias in rats exposed for 2 years to doses of 0.1–10r per day.

Lung tumours

Some strains of mice are particularly prone to the development of lung adenomas. These are benign tumours, alveolar in origin and are found in about 50 per cent of strain A mice at 12 months and in about 75 per cent at 18 months. These tumours are not lethal and are found incidentally at autopsies. Lorenz and his colleagues (1946) using chronic γ-irradiation at 8·8r per day were able to increase the incidence of lung tumours in strain A mice to 77 per cent at about 11 months as compared with 47 per cent in the control group. These authors pointed out that though this increase is significant for the period of observation, exposure to radiation may have done no more than hasten the formation of these adenomas without increasing the total incidence.

There is a suggestion that chronic γ-radiation at 0·11r per day may increase the incidence of lung adenomas in male LAF$_1$ mice, but decrease it in female mice (Lorenz and his colleagues, 1955) However, the life-span of the irradiated males was significantly longer than that of the non-irradiated controls, which may account for the observed increase in males. The life-span of irradiated and control females was not significantly different.

Henshaw, Riley and Stapleton (1947) also reported a decrease in lung adenoma incidence in female mice given single doses of γ-radiation or of fast neutrons, and that the decrease is more marked with higher doses, though they did not discuss the possible effect of decreased life-span in their experiments.

Lorenz and his colleagues (1947) observed several lung tumours in guinea-pigs following continued exposure for 3 or more years, at an unstated dosage level.

Histologically, these tumours resembled the lung tumours of mice. At the time of reporting no lung tumours had been observed in control guinea-pigs.

Bronchogenic cancers in rats several months after intubation of ^{144}Ce were reported by Lisco and Finkel (1949). This demonstrates that radioactive material deposited in the bronchi can produce carcinomas, but it must be noted that although no estimate can be made of the actual radiation dose given in these experiments it was sufficient to cause very severe lung damage.

Tumours of the ovary and testis

Ovary

In mice of some strains ovarian tumours occur spontaneously with a high frequency but are absent in others. X-irradiation appears to increase their incidence in all strains whether it is given as single doses or as fractionated or chronic treatment. The tumours are usually benign, hardly affect the life-span, and seldom metastasize. They are often multicentric in origin, frequently bilateral and the majority are of the granulosa cell type, though luteomas, tubular adenomas, haemangiomas, endotheliomas and even sarcomas have been reported.

Since the original discovery of the increase by x-irradiation in ovarian tumour incidence in 1936 (Furth and Furth, 1936; Furth and Butterworth, 1936) numerous investigators have confirmed their observations (Lick, Kirschbaum and Mixer, 1949; Henshaw, Riley and Stapleton, 1947). Lorenz and his colleagues (1947) found in experiments with LAF_1 mice that chronic exposure to 0·11r of γ-rays daily raised the incidence of ovarian tumours from 14 per cent in the untreated controls to 66 per cent. In a repeat experiment, however, with a larger group of animals (Lorenz and his colleagues, 1955) the incidence in the 101 controls was found to be only 4 per cent, while the incidence was 11 per cent in the 98 treated females.

TABLE IX

RESULTS OF IRRADIATION OF WHOLE BODY AND OF OVARIES ONLY IN MICE

Dose	No. of mice	No. with ovarian tumours	% incidence of ovarian tumours
	Whole-body irradiation		
0	24	0	0
12·5r	10	0	0
25r	10	0	0
50r	17	12	70
100r	10	9	90
200r	9	9	100
300r	8	8	100
400r	8	8	100
	Ovaries only irradiated		
50r	18	5	28
100r	10	3	30
200r	8	4	50
300r	9	9	100
400r	8	8	100

The effect of single doses of x-rays given to the whole-body or to the ovarian region only in LAF, mice was investigated by Deringer, Lorenz and Uphoff (1955), and their results are shown in Table IX.

The greater effectiveness of whole-body irradiation as compared with exposure of the ovarian region only indicates an influence of systemic factors. However, shielding of the ovaries (by exteriorization) during irradiation of the mouse with doses of up to 900r failed to induce any ovarian tumours (Deringer, Lorenz and Uphoff, 1955). Gardner (1950) had previously shown that oestradiol injection prevented ovarian tumour production in mice given whole-body exposure to 280 to 380r, whereas injection of testosterone propionate promoted the formation of ovarian tumours in irradiated mice (Gardner, 1953). Lick, Kirschbaum and Mixer (1949), using Bagg albino mice, found that irradiation of only one ovary with 200r failed to induce ovarian tumours when the other ovary functioned. If the other ovary was removed, an ovarian tumour developed in the irradiated ovary. Irradiation of both ovaries with 200r gave bilateral tumours in all 4 mice so treated. When irradiated ovaries were grafted into spayed mice or into irradiated mice (Kaplan, 1950), ovarian tumours developed in the grafts but failed to appear after grafting into untreated females, while non-irradiated ovaries grafted into spayed mice failed also to develop tumours. Thus direct effects and systemic factors including changed pituitary activity are necessary for the production of ovarian tumours.

Biskind and Biskind (1944) found that the grafting of ovaries into the spleen of spayed rats and mice caused tumours in the ectopic ovary. While normally the hormones produced by the ovaries pass through the circulation to the pituitary and thus damp down the output of gonadotropic hormones, the hormones of the ectopic ovary are carried to the liver with the splenic blood and there metabolized. Hence there is a low blood level of ovarian hormone which stimulates the pituitary to the production of gonadotrophic hormones. If oestrogens are administered to such spayed animals with ovarian grafts in the spleen the tumour formation is suppressed (Li and Gardner, 1949). On the other hand, the administration of gonadotrophins to mice has failed to induce ovarian tumours and their effect on rats is doubtful (Gardner, 1953).

While the Biskind phenomenon can be observed in rats and in mice, radiation has so far produced ovarian tumours in mice only.

In ectopic as well as in irradiated ovaries the ova disappear and follicle formation is initially inhibited. This is followed by invaginative tubular downgrowth of germinal epithelium which gives rise to the tumours Irradiated mice given large doses of oestrogens form anovular follicles instead of the tubular downgrowths and thus do not produce tumours (Gardner, 1955).

The ovarian tumours produce oestrogenic hormones and while in the ectopic ovaries these too reach the liver and are there metabolized, they reach the circulation from the irradiated ovarian tumours *in situ* and should thus damp down the pituitary output of gonadotrophins and thereby restrain tumour growth. This in fact does not happen and suggests that the pituitary does not respond to the hormones produced by the tumours. Alternately the tumours may be able to grow without further pituitary stimulation. This phenomenon as well as the necessity to irradiate the ovaries directly suggests that the mechanism by which ovarian tumours are produced by irradiation is different from the Biskind phenomenon.

Lorenz (1950) found that increasing the dose does not shorten the latent period for ovarian tumours as it does for leukaemia. Furthermore the threshold dose for ovarian tumour development is fairly sharp between 25 and 50r for LAF₁ mice (Deringer, Lorenz and Uphoff, 1955). Single exposures are apparently as effective as chronic exposure but the long latent period makes it difficult to be certain on this point.

Testis

Though the testis is one of the most radiosensitive organs in the body no reports of testicular tumours induced by radiation are mentioned in the literature. This is surprising as nodular hyperplasia of the interstitial tissue of the testis is not uncommon (Gardner, 1953). In rats, grafting of testes to the spleen of gonadectomized animals produced interstitial cell tumours and in mice these tumours have been produced in some strains by the administration of oestrogens.

Breast tumours

Most of the experimental work has been done with mice, where the occurrence of breast tumours is influenced by (1) genetic factors, as shown by the difference in incidence with strain, (2) hormonal factors, demonstrated by the greater incidence of breast cancer in intensely breeding as compared with virgin mice of the same strain, and (3) the presence or absence of the "milk factor" of Bittner which is carried over to suckling mice by the milk of their mothers or foster-mothers. All these factors must be considered when interpreting the results of radiation experiments. For instance, acute massive doses of radiation have reduced cancer incidence in some strains, presumably because of the sterilization of the mice (Lorenz and his colleagues, 1951). In C3Hb mice (which carry the milk factor) exposure to chronic irradiation has failed to influence the incidence of breast tumours. On the other hand, in LAF₁ mice and in C3Hb mice which are free of milk factor the incidence of breast tumours was increased by exposure to chronic irradiation from a radium source.

In LAF₁ mice exposed to 0·11–8·8r of γ-rays from radium daily the incidence of carcinomas increased to 15 per cent and that of sarcomas to 25 per cent while none were found in 29 controls. It is noteworthy, however, that the increase in breast tumours did not increase with increasing daily dose rates and that a dose rate of 2·2r daily was "optimal" in producing breast cancers (Lorenz and his colleagues, 1951).

In C3Hb mice (free of milk factor) the control incidence for breast tumours was 4 per cent in 160 virgin mice and 38 per cent in intensely breeding females. Irradiation of virgins with 8·8r of x-rays daily increased the tumour incidence to about 30 per cent in 91 mice for sarcomas and to about 20 per cent for carcinomas. In the intensely breeding controls the tumours are mainly adenocarcinomas, there were only 1 sarcoma and 1 carcinosarcoma in this series. Methyl cholanthrene painting of mice free of milk factor has produced, like irradiation, a similar diversity of sarcomas and carcinomas.

In irradiated mice breast tumours occur frequently in association with ovarian tumours and it is thought (Furth and Lorenz, 1954) that in spite of the sterilization by irradiation the subsequent production of hormone-secreting ovarian tumours may account for the increased incidence of breast tumours. However, in the

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a metastasizing renal carcinoma in a rat surviving for 10 months (Brecher, Cronkite and Peers, 1953). The morphology of the tumour tissue was epithelial in type and different from spontaneous renal tumours occurring in rats. Koletsky and Gustafson (1955) reported 8 clear-cell tumours of the kidney in 123 rats surviving for more than 6 months after a whole-body exposure to 660r of x-rays.

Liver tumours

Hepatomas are found in old mice in a frequency varying with the strain. They occur more often in males than in females and their incidence is reduced by castration and also by oestrogen treatment, while treatment of females with testosterone propionate does not affect the rate of hepatomas (Gardner, 1953). These tumours are invariably benign and are found incidentally at *post mortems*.

Deringer, Lorenz and Uphoff (1955) reported that in female LAF$_1$ mice x-ray doses of up to 400r to the whole body or to the ovaries only did not increase the incidence of hepatomas above the value of 11 per cent found in the control mice. Lorenz and his colleagues (1951) found only a slight increase above the control value of 8 per cent in female C3Hb mice given chronic whole-body irradiation at 8·8r per day.

Intestinal tumours

In rats protected against immediate irradiation effects by chemicals or parabiosis Brecher, Cronkite and Peers (1953) found 1 adenocarcinoma each of the duodenum, jejunum, ileum and colon using doses of from 700r to 1,000r. The duodenal and ileal tumour metastasized.

Tumours of the cerebro-spinal system

In rats protected by parabiosis or chemical methods and exposed to single whole-body doses of radiation 1 glioma was reported by Brecher, Cronkite and Peers (1953) and 1 meningioma by Finerty and his colleagues (1953).

Endocrine gland tumours

Most of the work on tumour induction in the pituitary and thyroid glands has been done with radioactive isotopes and is reviewed later.

Adrenal

Medullary tumours in old mice following castration by irradiation or by gonadectomy have been described by Smith and his colleagues (1949). These tumours are considered secondary to the effects of castration. Koletsky and Gustafson (1955) reported 4 adrenal tumours in 123 rats surviving for 6 or more months whole-body irradiation. Deringer, Lorenz and Uphoff (1955) reported 3 adrenal tumours in mice exposed to whole-body radiation, and 1 in mice whose ovaries had been irradiated.

Thyroid

Koletsky and Gustafson (1955) reported 2 adenocarcinomas and 16 benign adenomas of the thyroid in 123 irradiated rats as compared with 3 benign adenomas in 36 controls.

experiments of Lorenz and his colleagues (1955), exposure of LAF_1 mice to 0·11r of γ-rays daily produced 8 breast tumours of which 1 only was associated with an ovarian tumour. This suggests that in mice other factors than stimulation by ovarian hormones may be implicated in the production of breast tumours by radiation, and the same conclusion applies to rats in which breast tumours are not usually found in association with ovarian tumours.

In rats fibroadenoma is the most frequent tumour of the breast, and the incidence is increased by oestrogen treatment (Furth and Lorenz, 1954) and by whole-body irradiation (Brecher, Cronkite and Peers, 1953). Malignant breast tumours following single doses of whole-body radiation have been reported by Brecher, Cronkite and Peers (1953) and by Finerty and his colleagues (1953) using doses in the lethal range on protected animals. An increase in the incidence of benign mammary tumours in rats exposed for two years to chronic γ-radiation of 0·1r–10r per day was reported by Metcalf and Inda (quoted by Furth and Lorenz, 1954)

Lorenz (1950) reported an increased incidence of breast tumours in male as compared with female guinea-pigs exposed to chronic γ-ray irradiation, but did not mention the dose rate and duration of exposure.

Uterine tumours

Uterine tumours occur infrequently in rats and mice. In some stocks of rabbits uterine tumours are not infrequent in old animals. Lorenz (1950) found uterine tumours in 2 of 6 control rabbits while 11 of 12 rabbits exposed to daily doses of 1·1–8·8r of γ-rays from radium had uterine tumours. These occurred earlier than in the controls and the latent period in tumour formation decreased from about 52 months to 36 months with increasing dose rates. Furthermore in 7 of the irradiated rabbits the tumours metastasized while the tumours in the non-irradiated 'controls remained localized. The greater tendency to metastasis is attributed by Lorenz (1950), Kaplan and Murphy (1949) and others to the lowering of the immune responses of the animal by whole-body exposure.

In rats Brecher, Cronkite and Peers (1953) found a sarcoma of the uterus in one of their rats given 1,000r of x-rays in a single dose and protected against the immediate radiation effects by parabiosis.

In LAF_1 mice exposed to whole-body radiation Deringer, Lorenz and Uphoff (1955) found 7 tumours of the uterus in 72 animals given doses of from 12·5r to 400r. A sarcoma of the uterus was found in a female given 12·5r and 2 adenocarcinomas were observed after doses of 100r and 300r respectively. There was also a sarcoma in a mouse irradiated with 400r to the ovaries only. These tumours occurred in animals given doses which produced ovarian tumours, with the exception of the mouse given only 12·5r. Whether this increased incidence of malignant uterine tumours—fibromyomas only were observed in 1 control mouse out of 65—is due to the presence of the ovarian tumours remains to be investigated. Uterine tumours have been produced, although infrequently, by oestrogen treatment of mice (Gardner, 1953).

Kidney tumours

Whole-body irradiation with a single dose of 700r of x-rays to a rat protected by para-aminopropiophenone may have been responsible for the development of

TABLE X

Species	Field size or diameter	Dose in rad*	Source of radiation	No. at risk	No. of tumours		References
					Carcinoma	Sarcoma	
Mouse	4 × 1 cm.	4,600	^{32}P	45	3	2	Cloudman and his colleagues, 1955
"	4 × 1 cm	7,000–9,300	^{32}P	60	5	5	"
"	†	—	^{90}Y	20	9	4	"
"	1 cm.	8,000	Electron-beam 0.7 MeV	27	4	0	Glucksmann and Boag, 1951
Rat	2.5 cm	2,300	"	20	0	0	Glucksmann and Boag (unpublished)
"	2.5 cm	2 × 2,300†	"	28	2	5	"
"	2.5 cm	12,000	"	26	0	6	"
"	{ 2.5 cm ‡ / 10 cm³	12,000† / 2,000 }	"	24	2	13	"
"	35 cm³	5,500	Sr^{90}	71	6	2	Passonneau and Hamilton, 1951
"	35 cm.²	8,200	"	73	4	9	"
"	35 cm³§	8,200	"	74	1	0	"
"	35 cm³¶	8,200	"	77	3	4	"
"	35 cm³**	8,200	"	58	4	3	"

* Where necessary converted from dose given in rep
† Implanted as subcutaneous pellets (total activity 11 μc)
‡ Two fractions at an interval of 2 months
§ Ten beads containing ^{90}Sr distributed over 35 cm² area.
¶ Twenty beads containing ^{90}Sr distributed over 33 cm² area.
** Fifty beads containing ^{90}Sr distributed over 33 cm² area.

of radiation is illustrated in the work of Passonneau and Hamilton (1951). Uniform irradiation of a given field is more efficient in tumour production than concentration of the same amount of radioactivity into a number of smaller sources. The necrotizing action of radiation from a few highly active beads may account for the reduction in the incidence of tumours.

In the work of Glucksmann and Boag with electron-beams it was found that a dose of 4,600 rad given in two fractions two months apart produced about as many tumours as a single dose of 12,000 rad.

Combination of radiation and chemical carcinogens

The interaction of irradiation and chemical carcinogens was first investigated by Mottram (1938) After 20 applications of 0 5 per cent benzpyrene to the back of mice over ten weeks, irradiation with doses 800–2,800r was followed by the appearance of warts and carcinomas, while a dose of 6,000r suppressed cancer formation. Shubik and his colleagues (1953) exposed mice to a single dose of 800 rep of β-rays and followed this treatment with croton oil painting twice weekly. After an average latent period of 3.5 months 13 tumours appeared in 6 of 17 mice exposed to risk These tumours at the time of reporting had neither regressed nor become malignant.

Cloudman and his colleagues (1955) studied the effect of a single application of 0.6 per cent methylcholanthrene in benzene, alone and after exposure to a single dose of β-rays (Table XI) The combined application resulted in a significant increase in the number of tumours.

Data of Glucksmann and Boag (unpublished) are also shown in Table XI, they found an enhancing effect for a combination of electron-beam exposure and painting with a weak carcinogen. However, exposure to an electron-beam 15 months before a weekly application of a strong carcinogen (9 : 10 dimethyl 1 · 2 benzanthracene) was found to decrease the incidence of tumours below that

Pituitary

Though changes in the pituitary secondary to castration by irradiation have been described, pituitary tumours following whole-body irradiation are not recorded. The pituitary tumours following administration of isotopes are discussed later.

CARCINOGENIC EFFECT OF LOCALIZED IRRADIATION

Introduction

Most of the early experimental work on radiation carcinogenesis was done with localized radiation, mainly by the implantation of radium sources in various forms. This work, reviewed by various authors (Lacassagne, 1929, Furth and Lorenz, 1954) showed the carcinogenic effect of localized irradiation and, particularly in the studies of Ross (1936), demonstrated that the same radiation exposure would produce different types of tumour according to the tissue into which the source was implanted. Using radium tubes implanted into rabbits Ross obtained an osteogenic sarcoma from a tube in periosteal tissue, spindle-cell sarcomas in connective tissue, a myxo-sarcoma in subcutaneous tissue and squamous-cell carcinomas from tubes in the dermis.

From most of these experiments, however, little information can be gained concerning the levels of radiation dosage, except that they were sufficiently high to cause a severe degree of local tissue damage.

This earlier work has been reviewed by others and the more recent studies are considered here These comprise the carcinogenic effects of local irradiation on skin with a comparison of the histological processes in radiation and chemical carcinogenesis, the carcinogenic effects of the bone-seeking radioactive isotopes, pituitary and thyroid tumours arising after thyroid irradiation by ^{131}I, and tumours following administration of Thorotrast.

Skin tumours—tumour incidence

The first experimental proof of the carcinogenic action of penetrating radiations was based on partial body exposures of the skin of experimental animals and consisted in showing ulcerative and later malignant changes present only in the irradiated region. Lacassagne (1945a) pointed out that studies with x-rays or γ-rays of deep penetration proved very difficult in small animals as it was almost impossible to avoid giving doses to particularly sensitive organs such as the intestine, causing the death of the animal before tumours can develop. The use of electron-beams and of β-rays on small animals has overcome this difficulty.

Partial body irradiation with electron-beams (Glucksmann and Boag, 1951, 1954), and with β-rays (Cloudman and his colleagues, 1955, Passonneau and Hamilton, 1951) has produced carcinomas and sarcomas in mice and rats. This work is summarized in Table X.

The latent period in tumour formation in the studies listed in Table X varied from 10 months to 23 months and longer.

Field size is of great importance and this probably explains the difference in tumour incidence shown in Table X as compared with that in Table VII which refers to whole surface exposure. The role of field size and uniform distribution

to act by producing conditions in which the repair tissue undergoes a malignant change.

Carcinogenic effects of bone-seeking radioactive isotopes

Tumour incidence

One particular aspect of experimental work on partial body radiation deals with the effects of the bone-seeking radioactive isotopes. The carcinogenic effect of ingested radium was demonstrated in the dial painters. Since other bone-seeking radioactive materials are formed in nuclear energy processes it has become a matter of the greatest importance to determine the extent to which these new materials also have carcinogenic properties, and to learn as much as possible about the process of bone tumour production with these materials. For this reason animal experiments have been carried out with a number of the bone-seeking materials, the main β-emitters studied being ^{32}P, ^{89}Sr, ^{90}Sr and ^{91}Y (Koletsky, Bonte and Friedell, 1950, Moller, 1954, Finkel, Lisco and Brues, 1955), while most of the work with α-emitters has been carried out with ^{226}Ra and ^{239}Pu (Dunlap and his colleagues, 1944, Finkel, 1953).

Distribution within bone

The metabolism of the bone-seekers has been shown by autoradiography of the bone to follow. one of two main patterns (Hamilton, 1948) The elements strontium and radium are like phosphorus and calcium found in the mineralized part of bone. The other class of elements, including plutonium, thorium, neptunium, cerium, americium and curium, do not take part in the mineralization process but remain localized in the organic material surrounding the bone shaft and the trabeculae. Within this second class certain differences have been observed (Hamilton, 1948). Cerium, americium and curium are localized not only on the bone surface but are also irregularly distributed within the shaft of the bone, presumably on the linings of the blood vessels. The autoradiographic studies of Vaughan (personal communication) have shown that yttrium, which was originally considered to have the same pattern of distribution in bone as plutonium, appears to be mainly concentrated at resorption sites.

The radiation dose and dosage rate in bone will clearly depend on the pattern of distribution of the isotope in the bone, and on its retention within the bone, which in turn depends on the specific chemical form, the route of administration and species, age and other factors influencing bone metabolism. The quantitative data on carcinogenesis is considered in relation to individual isotopes.

Strontium.—Radioactive strontium (either as ^{89}Sr or ^{90}Sr in equilibrium with ^{90}Y) has been used most frequently for carcinogenic studies since, for a given carcinogenic effect, it produces less general radiation effects than, for instance, plutonium or phosphorus. This is due to its high retention in bone (greater than 70 per cent) and low uptake in soft tissues.

Finkel, Lisco and Brues (1955), summarized experiments with ^{90}Sr on a total of 3,083 mice and reported a total of 427 malignant tumours in 191 mice. The majority of the tumours (92 per cent) were osteogenic sarcomas.

CF 1 female mice and ABC male and female mice were used in these experiments There were strain and sex differences in the incidence and location of the tumours,

TABLE XI

Species	Dose in rad*	Carcinogen	No at risk	No. of tumours		References
				Car-cinoma	Sar-coma	
Mouse	4,600	None	45	3	2	Cloudman and his colleagues (1955)
,,	None	1 × MCA†	30	5	0	,,
,,	4,600	1 × MCA	29	9	3	,,
Rat	2,300	None	20	0	0	Glucksmann and Boag (un-published)
,,	None	DBA‡ weekly	19	0	0	,,
,,	2,300	DBA weekly	26	0	3	,,
,,	2,300	DMBA§ weekly	15	3	6	,,
,,	None	DMBA weekly	48	43	16	,,

* Where necessary converted from dose given in rep
† MCA = 0 6 per cent solution of methycholanthrene in benzene.
‡ DBA = 1 per cent solution of 1 2 5 6 dibenzanthracene in acetone
§ DMBA = 1 per cent solution of 9 10 dimethyl 1 2 benzanthracene in acetone

obtained with the carcinogen alone, nor to alter the proportion of sarcomas to carcinomas.

If the histogenesis of cancers produced by the painting of chemical carcinogens such as benzpyrene (Glucksmann, 1945) or dimethylbenzanthracene is compared with that due to the external irradiation with electron-beams the following points of difference emerge:

(1) Potent chemical carcinogens produce skin cancers in almost all mice in 3-5 months while radiation induced 10-15 per cent skin cancers in about 15-20 months. In rats painting with carcinogens is followed by the appearance of cancers in 98 per cent of treated animals after a median latent period of about 10 months. Irradiation produced malignant tumours in 50 per cent of rats after a median latent period of 23 months.

(2) Sarcomas and carcinomas appear in different proportions in chemical and radiation-induced lesions In mice practically all tumours are carcinomas after painting, while only 36 per cent of tumours induced by β-rays are carcinomas, the majority being sarcomas For rats the comparable figures are 73 per cent for carcinomas following painting and 14 per cent following exposure to electron-beams In the combined treatment with radiation and chemicals the percentage of carcinomas in mice increased to 78 per cent and in rats to 25 per cent.

(3) With chemical carcinogens the primary effects are growth-stimulating on both epidermis and hair follicles, and this effect presumably accounts for the greater efficiency and shorter latent period in tumour development, and also for the greater proportion of carcinomas, a number of which originate in the hair follicles The transient epilation is due to growth-stimulation of the epithelial hair sheath With irradiation the primary effects are injurious, leading to the shedding of the epidermis and to the destruction of hair follicles, followed by the appearance of inflammatory cells and fibroblasts in the dermal scar. Malignancy supervenes late in the process of regeneration after the repeated breakdown of the scars, which accounts for the long latent period The absence of the hair follicles and the atrophy of the epidermis on the one hand, and the appearance of numerous connective tissue cells on the other hand account for the increased proportion of sarcomas and the decrease of carcinomas

In summary, the chemical carcinogens are more effective and quicker in the production of skin cancers than irradiation because they stimulate directly the treated cells and their offspring, while irradiation is primarily injurious and appears

injected dose, corresponding to about 20 μc per gramme, which in a uniform medium would yield a dosage rate of over 1000r per day. The concentration of activity in this volume of tissue will reduce with time, but a very large radiation dose must have been received by the tissue before the tumour was observed.

Phosphorus.—Koletsky and his colleagues (1950) reported experiments with ^{32}P on rats, using single doses of 4.5 μc per gramme and repeated doses of 1.5 μc/gramme up to a total of 9 or 12 μc/gramme. Malignant tumours appeared in 9 out of 19 rats surviving the single dose, and in 4 out of 15 rats receiving the repeated injections. All were osteogenic sarcomas with the exception of 3 squamous-cell carcinomas of the face.

In a later paper Koletsky (1954) reported a further study with lower doses of ^{32}P, giving single doses of 0.25–3.5 μc per gramme, to Wistar rats. Apart from one rat with lymphosarcoma the 43 animals receiving 0.25 or 0.5 μc per gramme, and surviving 8 months did not develop tumours. With doses from 1.0 to 3.5 μc per gramme tumours appeared in 22 to 50 per cent of the animals. The authors stated that the latent period up to the time of detection of the neoplasm appeared to be lengthened when the dose of ^{32}P was lowered, being 19, 14 and 10.5 months respectively for animals given 1, 2 and 3 μc per gramme of ^{32}P. As in the earlier study, the majority of tumours were osteogenic sarcomas together with some cases of squamous-cell carcinoma of the face. The exact origin of this type of tumour was not determined, but it probably arose close to bone in the epithelium lining the accessory nasal sinuses, nasopharynx, and roof of the mouth.

The data are not available to make an estimate with any precision of the dose received by the bone. However, in view of the known concentration of administered ^{32}P in bone, it is likely that an injected dose of 1 μc per gramme would give an initial radiation dose to the bones greater than 100r per day.

A study of the carcinogenic action of ^{32}P on mice is reported by Møller (1954), using much smaller doses of ^{32}P. Two groups of mice were used, one group given a single injection of about 0.2 μc per gramme and the other group given eight injections of about 0.2 μc per gramme at six-weekly intervals. 385 injected mice and 203 control mice were used. The incidence of leukaemia and of subcutaneous tumours was not appreciably different in the two groups, but in the irradiated group 3 bone tumours were observed, while none were found in the controls and, in fact, have never been reported in this strain (ST/Eh).

The bone tumours appeared as follows:

Spine	15 months after injection	Single dose
Jaw	27 months after injection	Single dose.
Jaw	21 months after first injection	Repeated dose

Data are not available for determining with any precision the value of the dose received by the mouse bones from an injection of 0.2 μc per gramme of P^{32} but the total dose received by certain parts of the bone may be as high as several hundred roentgens

Plutonium and related elements.—The most efficient carcinogen amongst the radioactive bone seekers is ^{239}Pu. Quantitative data is given by Finkel (1953). A dose of 1 μc per kilogram of ^{239}Pu led to the appearance of bone tumours in over 40 per cent of CF 1 female mice surviving the latent period of 180 days. The corresponding value for control mice was about 3 per cent.

the CF 1 female mice showed more tumours (54 per cent) than the ABC females (28 per cent), and the ABC females more than the ABC males (18 per cent).

The location of the bone tumours was as follows:

Site					Percentage
Long bones—femur and tibia	.		.	..	57·6
humerus	..	.		..	4 0
Spine	..	..	.	..	25 7
Skull	..	..	.	..	7 5
Other bones	..	..	..	..	5 0

In these experiments various dosages of ^{89}Sr, single and repeated, were used. Tumours were not found before 180 days. For a single intraperitoneal injection the rate of tumour production increased up to 2.5 μc per gramme, and then apparently decreased. With monthly repeated doses the tumour incidence increased up to 1.0 μc per gramme, the highest monthly dose given. Repeated doses of 0.5–1.0 per gramme per month were more efficient, while repeated doses of 0.1 and 0.2 μc per gramme per month were less efficient than the same total dose given in a single injection, implying that recovery factors are operative with the lower levels of dosage.

The authors concluded that a single dose of 0.2 μc per gramme and monthly repeated doses of 0.1 μc per gramme undoubtedly induce bone tumours, but that with lower doses the evidence for increased tumour incidence is merely suggestive. Doses of ^{89}Sr between 0.001 and 0.005 μc per gramme do not appear to have an effect on the incidence of bone tumours in mice.

It is of interest to make a rough estimate of the dose to bone in this experiment. Following a dose of ^{89}Sr of 0.2 μc per gramme body-weight, the activity concentration in the bony part of the skeleton will be 1.4 μc per gramme, assuming that 70 per cent of the strontium is taken up by the skeleton, and that the skeleton represents one-tenth of the total body-weight. A concentration of 1.4 μc per gramme of ^{89}Sr would yield a dose rate of about 50r per day in a medium of linear dimensions greater than the range of the strontium electrons. The fact that the thickness of mouse bones is small compared with the mean range of the strontium electrons will reduce the mean dose to bone below the value given above, but on the other hand there will be local concentration of strontium in growing parts of the bone which will lead to an increase in dose rate. Certainly, with the latent periods observed in these experiments, parts of the bone may well have received doses of thousands of roentgens prior to the appearance of the tumours.

Owen, Jowsey and Vaughan (1955) have carried out detailed studies of the uptake and localization of radioactive strontium (^{90}Sr) in the bones of the rabbit. In a series of experiments (1955), bone changes were studied in eight young rabbits given single doses of 0.5 to 1.1 μc per gramme and surviving 180 days or more Three animals showed bone tumours, two in the skull and one in the left tibia (animal died at 180 days). The tumour in the tibia developed calcified cartilage containing ^{90}Sr, which apparently was the remnant of the epiphyseal plate at the time of injection Previous distribution studies had shown that the concentration of ^{90}Sr in the epiphyseal plate of the tibia in rabbits of the appropriate age is very much greater than in most other parts of the bone. At 24 hours after injection the uptake in the epiphyseal plate is 4.4 per cent per gramme of the

either to insufficient concentration of thyroid hormone or to a diminished responsiveness of the dependent tumour cells (Furth, 1954). After several transplantations into conditioned hosts the tumours tend to become independent (autonomous) and grow in normal animals, where their growth can actually be stimulated by thyroid hormone (Furth, 1953).

Thyroid gland remnants left after incomplete surgical or ^{131}I-thyroidectomy in mice tend to undergo an adenomatous hyperplasia (Dent, Gadsden and Furth, 1955).

The thyroid tumours produced experimentally in rats by ^{131}I treatment also tend to start as dependent neoplasms which grow only in conditioned hosts and to acquire autonomy after several transplantations (Morris, 1955).

The pituitary tumours in mice treated with ^{131}I are thus due to indirect radiation effects through the destruction of the thyroid while the thyroid tumours in rats may be due to a more direct action on the thyroid epithelium possibly combined with stimulation by thyrotropic hormones.

Tumours following Thorotrast administration

A special type of non-uniform irradiation is provided by Thorotrast, a colloidal solution of thorium dioxide which has been used as a radiographic contrast agent. Clinically it has been found sometimes to have serious late effects, including a small number of reported cases of malignancy. Its carcinogenic properties have been investigated experimentally by a number of workers, using various routes of administration—subcutaneous, intraperitoneal and intravenous. The experimental work, together with the clinical findings on malignancy, have been reviewed by Guimaraes, Lamerton and Christensen (1955). Following subcutaneous and intraperitoneal injection the most common type of tumour is a spindle-cell sarcoma. Work on rabbits and mice using intravenous injection (Johansen, 1955, Guimaraes, Lamerton and Christensen, 1955) has demonstrated the production of a number of reticulo-endotheliomas in the liver and spleen, and also of haemangioendotheliomas in the spleen.

Thorium itself has a very long half-life and emits α-particles. Its disintegration products emit both α- and β-particles. By whatever route Thorotrast is administered there will be a certain degree of aggregation of the particles in the body, so that the tissues will receive chronic α-irradiation (together with some β-irradiation), but, in general, with a non-uniform dosage distribution throughout the tissue, depending on the distribution and size of the aggregates. Only in a few cases has any estimation been made of the radiation dosage delivered in experiments where malignancies resulted. These estimates have been based on the autoradiographic studies made by Rotblat and Ward (1953) using human tissues containing known concentrations of Thorotrast. Johansen (1955) estimated that the mean α-dose in the livers and spleens of his rabbits, a number of which developed tumours, mainly reticulo-endotheliomas (reported to be of multifocal origin in the liver, spleen and lung) from 2 to 4 years after injection, was greater than 1·3 rep per day, so that in the time before the observation of tumours a mean dose of greater than 1,000 rep would have been accumulated. Local dosage is possibly much higher than this in view of the non-uniformity of distribution, and also the high relative biological efficiency of α-particles as opposed to x-rays or β-particles will increase the effective dose.

Since plutonium localizes only on the surface of bones and the α-radiation emitted has a limited range (about 30 microns) the volume in which the absorption of radiation energy occurs is limited, and thus the radiation dose levels may be high.

General consideration of carcinogenic action of bone-seekers

Emphasis has been placed on the radiation dose received by bone tissue from amounts of isotopes which have a carcinogenic effect. It was seen that this dose may be large, of a magnitude sufficient to cause considerable tissue damage.

Bone size as well as metabolic activity can affect the level of the radiation dose in localized regions, and these factors must be considered in any study of the influence of strain, sex and species or the incidence and distribution of bone tumours following ingestion or injection of bone-seeking radioactive materials.

Pituitary and thyroid tumours produced by radioactive iodine

Spontaneous thyroid tumours occur rarely in rats and mice (Morris, 1955) but can be induced by goitrogens and by chemical carcinogens. Administration of large doses of ^{131}I may also be followed by the appearance of thyroid tumours in rats and of pituitary tumours in mice.

Goldberg and Chaikoff (1952) observed 8 malignant and 10 benign thyroid tumours in 9 of 25 rats 1½–2 years after an injection of 400 μc of ^{131}I. As the remaining thyroid gland epithelium did not indicate unduly high levels of thyrotropic hormone secretion, a direct effect of the radiation on thyroid tissue was presumed to be responsible for the development of the thyroid neoplasms.

Doniach (1953) investigated the effect of combining methylthiouracil with ^{131}I treatment. In a group of 20 rats given methylthiouracil and a dose of 30 μc ^{131}I he obtained thyroid carcinomas in 5 rats and thyroid adenomas in all the rats. None of the rats given methylthiouracil alone produced tumours. The relatively low radiation dosage was found to interfere with thyroid function, to lower the concentration of thyroid hormone and thus to stimulate the secretion of thyrotropic hormone. The subsequent stimulation of the thyroid epithelium appeared to create conditions favourable for tumour production by the goitrogen.

In mice dosages of 40–50 μc per gramme of ^{131}I failed to induce thyroid tumours but led to the appearance of pituitary tumours (Gorbman, 1949). Furth and Burnett (1951) reported that all C57 black mice surviving for 13 months or more after an injection of 200–400 μc of ^{131}I developed pituitary tumours. The doses were sufficient to destroy most or all of the thyroid tissue, and the more complete the thyroidectomy by ^{131}I the shorter was the latent period and the more rapid the growth of the pituitary tumours. Later Furth (1954) and Dent and his colleagues (1955) reported that complete, or almost complete, surgical thyroidectomy also produced pituitary tumours in mice, as did treatment with propylthiouracil (Moore and his colleagues, 1953). Induction of these tumours can be prevented by treatment with thyroid hormone (Furth, 1954).

Pituitary tumours can at first be transplanted only to thyroidectomized mice in which thyroidectomy has been achieved by surgery or by ^{131}I (Dent, Gadsden and Furth, 1955). On treatment with thyroid hormone these dependent (conditional) neoplasms can be restrained, but not made to regress, which may be due

Experimental work

The importance of species and sex variation is evident in all the experimental work done. The types of tumour produced and their rate of appearance, for the same radiation conditions, are found to differ greatly between different species and also, in some cases, between different strains of the same species. This demonstrates the importance of systemic factors in radiation carcinogenesis and at the same time makes it very difficult to use animal results for the estimation of carcinogenic risk in man

The levels of radiation dose required for carcinogenesis in experimental work are found to vary over a wide range, depending on the relative importance of the effects of local tissue damage, of predisposing tissue factors and of systemic effects. Where local tissue effects are the main factor, as in the case of tumours following localized irradiation of skin and bone the doses required are of the order of one to several thousand roentgens. This applies whether the exposure is from an external source or is the result of internally deposited radioactive materials. If there are predisposing tissue conditions, produced for instance by the presence of inflammatory agents the dose required may be reduced to a few hundred roentgens.

In those cases where hormonal influences play a large part the dose will depend on the radiosensitivity of the glands involved and their relation to other endocrines. Ovarian tumours in mice can be produced by doses of radiation as low as 50r, if both ovaries are exposed, though radiation-induced ovarian tumours have not been reported in any other species. On the other hand, to produce pituitary tumours in mice by ^{131}I injection, the necessary dose to the thyroid is of the order of tens of thousands of roentgens.

One of the most interesting questions that arises in connexion with systemic effects and the radiation dose necessary for carcinogenesis is the relative effectiveness of localized and whole-body irradiation in producing various types of tumours.

In the case of lymphatic leukaemia in certain strains of mice it has been found that the incidence after irradiation is greatly decreased by the shielding of a small part of the thigh. It is not known whether there are other types of tumours for which a similar effect may be found. Several types of tumour have been reported by various workers using single doses of whole-body irradiation with doses of less than 1,000 roentgens, and it will be of interest to determine whether or not a considerably higher dose is needed for carcinogenesis when a localized irradiation is given.

Effect of dose and dosage rate

With regard to the questions concerning the influence on tumour incidence of dose, dosage rate, fractionation and protraction, only a few very general observations can be made on the basis of the experimental data.

None of the animal experiments have indicated a linear relationship between tumour incidence and dose. In the special case of tumours which are to a very large extent dependent on hormonal disturbance, such as ovarian or pituitary tumours in mice, there is good evidence for the presence of an absolute threshold, corresponding to the dose required to produce the necessary degree of hormonal disturbance, which will vary greatly with the type of tumour. It is not possible to make any statement about the existence of a threshold in other cases, but it can

It is possible, however, that the Thorotrast tumours do not represent a simple case of radiation carcinogenesis, as the effects of the aggregates may also play a part. This was the conclusion of Selbie (1938) who obtained tumours in a high proportion of rats and mice following subcutaneous injection of Thorotrast (for groups of 60 rats and 60 mice surviving 52 weeks, 58 per cent of the rats and 26 per cent of the mice showed tumours). He observed fibroblastic proliferation in many of the tumour rats, which suggested that tumours are formed in those animals which give a vigorous inflammatory reaction to the presence of Thorotrast. The action of inflammatory processes in increasing the carcinogenic action of localized radiation has already been mentioned and will be discussed in more detail.

In the present state of knowledge it is difficult to draw conclusions concerning the mechanism of carcinogenesis by Thorotrast. The particular need is for a study of the extent to which the physical presence of the Thorotrast aggregates affects the tissues.

DISCUSSION

Human experience

In spite of the large amount of data reported on radiation-induced cancer in man and in animals the mechanism of radiation carcinogenesis and the magnitude of the risk to man from a given type of radiation exposure is still unknown.

The human data provides little material on which to formulate theories. Not only is the data on the populations at risk either scanty or non-existent, but in most cases knowledge of the radiation dose and dosage distribution is very inadequate. In certain of the groups of suspected radiation-induced cancer in man, such as the lung cancer of the uranium miners at Schneeberg and Joachimsthal, other factors are present which may themselves lead to cancer production or profoundly modify the carcinogenic effect of radiation. The main finding from the human experience with localized radiation is that the radiation doses required for carcinogenesis are of a magnitude that produce severe tissue damage. Experience with skin and bone tumours indicates that a severe degree of tissue damage precedes the appearance of radiation-induced cancer. This, together with the observation that certain inflammatory processes increase the susceptibility of tissue to malignancy following radiation exposure suggests that the important parameter to consider is not the dose of radiation given, but the degree and type of general tissue damage produced, which initiates the repair processes.

The development of cancer is, however, by no means the invariable result of severe tissue damage produced by radiation. On the contrary, radiotherapeutic experience suggests that it is a rare sequel. For instance, osteosarcoma of the rib following breast irradiation has been reported only twice in spite of the popularity of this technique.

The induction of malignancy in the thyroid following thymic irradiation in infancy and of osteosarcoma after radium ingestion and administration need special consideration. In the case of the thyroid tumours hormonal influences may play a part. For the bone tumours it has been shown that the radium is concentrated in small areas of bone, so that the local radiation dose to vulnerable structures is high. Also the radiation emitted includes α-particles, which are much more effective than x- or γ-radiation in producing most types of tissue damage.

More work must be done before it will be possible to discuss with any confidence the mechanisms of radiation carcinogenesis. If one line of investigation more than another appears to be fruitful at the present time it is the careful study of the changes occurring between radiation exposure and the appearance of the tumour.

REFERENCES

Abrahamson, L., O'Connor, M. H., and Abrahamson, M.L. (1950). *Irish J. Med. Sci.,* 293, 229.

Anderson, N P., and Anderson, H. E. (1951). *Arch. Derm. Syph.,* 63, 586.

Aub, J. C , Evans, R. D., Hempelmann, L. H., and Martland, H. S. (1952). *Medicine,* 31, 221.

Auerbach, D , Friedman, M., Weiss, L., and Amory, H. I. (1951) *Cancer,* 4, 1095.

Bale, W F., and Shapiro, J. (1955). *Paper P/76, Vol. 13, Proceedings International Conference Peaceful Uses of Atomic Energy.* Geneva.

Biskind, M. S , and Biskind, G. S. (1944). *Proc. Soc. Exp. Biol.,* 55, 176.

Bloch, B (1924). *Schweiz. Med. Wschr.,* 57, 857.

Brecher, G., Cronkite, E. P., and Peers, J. H. (1953). *J. Nat. Cancer Inst.,* 14, 159.

Brues, A. M (1951). *Advances in Biological and Medical Physics,* Vol. 2, page 171. New York; Academic Press.

—— (1954). *Advances in Cancer Research,* 2. New York; Academic Press.

—— Sacher, G A., Finkel, M., and Lisco, H. (1949). *Cancer Res.,* 9, 545.

Burrows, H., Mayneord, W. V., and Roberts, J. E. (1937). *Proc. Roy. Soc. B,* 123, 213.

Cahan, W. G., Woodard, H. Q , Higinbotham, N. L., Stewart, F. W., and Coley, B. L. (1948). *Cancer,* 1, 3.

Cilley, E. I. L , Kirklin, B. R., and Leddy, E. T. (1935). *Amer. J. Roentgenol.,* 33, 390.

—— Leddy, E. T., and Kirklin, B. R. (1935a). *Ibid.,* 33, 787.

—— —— (1935b). *Ibid.,* 34, 241.

Clark, D E (1955). *Paper P/242, Vol. 11, Proceedings International Conference Peaceful Uses of Atomic Energy.* Geneva.

Cloudman, A M., Hamilton, K. A., Clayton, R. S , and Brues, A. M. (1955). *J. Nat. Cancer Inst.,* 15, 1077.

Coenen, H. (1909) *Berlin Klin Wschr.,* 46, 292.

Court Brown, W. M., and Abbatt, J. D. (1955). *Lancet,* 1, 1283.

Deller, P (1951). *Ibid.,* 1, 1159.

Dent, J. N., Gadsden, E. L , and Furth, J. (1955) *Cancer Res.,* 15, 70.

Deringer, M. K., Lorenz, E., and Uphoff, D. E. (1955). *J. Nat. Cancer Inst.,* 15, 931.

Deuticke, P (1939) *Z. Klin. Chir.,* 169, 214.

Doniach, I. (1953) *Brit. J. Cancer,* 7, 181.

Duffy, B J. Jnr., and Fitzgerald, P. J. (1950). *Cancer,* 3, 1018.

Dunlap, C E., Aub, J. C , Evans, R. D., and Harris, R. S. (1944). *Amer. J. Path.,* 20, 1.

Evans, R. D. (1950). *6th International Congress Radiology,* p. 8.

—— and Goodman, C (1940). *J. Ind. Hyg. Toxicol.,* 22, 89.

Finerty, J. C , Binhammer, R T., Schneider, M , and Cunningham, A. U. B. (1953). *J. Nat Cancer Inst.,* 14, 149.

Finkel, M. P. (1953). *Proc. Soc. Exp. Biol.,* 83 494.

Frieben, (1902). *Fortschr. Rontgen.,* 6, 106.

Furth, J. (1953). *Cancer Res.,* 13, 477.

—— (1954). *J. Nat. Cancer Inst.,* 15, 687.

—— and Burnett, W. T. Jnr. (1951). *Proc. Roy. Soc. Exp. Biol.,* 78, 222.

be said that the incidence of tumours is much greater above a certain dosage level (which depends on experimental conditions) and which in many cases, such as for skin and bone, corresponds to the production of a severe and lasting degree of tissue damage.

In the case of local irradiation the rate of tumour incidence does not necessarily increase indefinitely with dose. Several experiments, for instance the work on ^{89}Sr in mice, have shown a decrease in tumour incidence above a certain dosage level, presumably because the processes of regeneration have been interfered with too seriously.

Concerning the effect of dosage rate, protraction and fractionation the animal data at present available can give an answer only in one or two special cases. Even when the comparative data are available a quantitative comparison is frequently difficult to make, because account must be taken of a number of factors, including latent period and also "wasted" radiation. For skin tumours in rats produced by whole-body β-irradiation, single doses are considerably more effective in terms of the same total dose than chronic irradiation. The work of Lorenz, using chronic radiation at various dosage rates (0·11-8·8r per day) shows that, in general, the higher dosage rates are the more effective, though there is a suggestion of an optimal dose rate in certain cases, such as for breast tumours in mice. An optimum fractionation and spacing has been reported for the induction of lymphoma in mice after whole-body irradiation, and for the induction of skin tumours with localized irradiation.

Mechanisms of radiation carcinogenesis

The evidence at present available suggests that radiation-induced cancer arises from a tissue environment that has suffered severe disorganization, either as the result of local radiation damage, of hormonal disturbance, or of other physiological change arising from radiation exposure. It seems likely that a certain degree of disorganization in the tissue environment is essential for the appearance of radiation-induced cancer. It is not an unreasonable assumption that cancer develops as a result of the processes of repair, or attempted repair, consequent on the tissue disturbance; there is in some cases strong histological evidence for this.

The question arises whether the development of cancer demands a specific type of cellular change produced by the irradiation, as well as disturbance of the environment. The experiments on tumour induction in the thymus indicate that direct irradiation of the thymus is not essential. On the other hand, ovarian tumours in mice do not arise following irradiation unless both ovaries in the normal animal have been irradiated, but in this case the process is very complicated by hormonal disturbances.

There is histological evidence suggesting that malignancy has in certain cases developed in repair tissue outside the radiation field, but it is obviously not possible to prove that any given cell or its predecessors have not been irradiated. If the cancer develops from a given cell in which radiation has produced a particular change then, in view of the long latent period that can elapse between irradiation and the appearance of the tumour, it is likely that the change produced by radiation is only one of a series of changes which will eventually result in a malignant cell. Such a "multistage" theory has been applied to spontaneous as well as radiation-induced cancer by a number of workers.

Lacassagne, A. (1954) *Acta Union Intern. Contre le Cancer*, **10**, 56.
—— and Vincent, R. (1929). *Compt. rend. Soc. Biol.*, **100**, 249.
Lange, R. D., Moloney, W. C., and Yamawaki, T. (1954). *Blood*, **9**, 547.
Leddy, E. T., and Rigos, F. J. (1941). *Amer. J. Roentgenol.*, **45**, 696.
Li, M. H., and Gardner, W. U. (1949). *Cancer Res.*, **9**, 35.
Lick, L., Kirschbaum, A., and Mixer, H. (1949). *Ibid.*, **9**, 532.
Lisco, H., and Finkel, M. P. (1949). *Fed. Proc.*, **8**, 360.
Looney, W. B. (1955). *J. Bone Jt. Surg.*, **37A**, 1169.
—— (1956). *Ibid.*, **38A**, 160, 392
Lorenz, E. (1944). *J. Nat. Cancer Inst.*, **5**, 1.
—— Heston, W. E., Deringer, M. K., and Eschenbrenner, A. B. (1946). *Ibid.*, **6**, 349.
—— Eschenbrenner, A. B., and Deringer, M. K. (1947). *Radiology*, **49**, 274.
—— (1950). *Amer. J. Roentgenol.*, **63**, 176.
—— Eschenbrenner, A. B., Heston, W. E., and Uphoff, D. (1951). *J. Nat. Cancer Inst.*, **11**, 947.
—— Hollcroft, J. W., Miller, E., Congdon, C. C., and Schweistaal, R. (1955). *Ibid.*, **15**, 1049
Ludin, M (1934). *Acta Radiol.*, **15**, 553.
March, H. C. (1944). *Radiology*, **4**, 275.
—— (1950). *Amer. J. med. Sci.*, **220**, 282.
Marie, P., Clunet, J., and Raulot-Lapointe, G. (1910). *Bull. Ass. franç. cancer*, **3**, 404.
Marquès, P., Planel, H., and Hermous, G. (1952). *Ibid.*, **39**, 214.
Martland, H. S., Conlon, P., and Knef., J. P. (1925). *J. Amer. med Ass* , **85**, 1769.
—— (1931). *Amer. J. Cancer*, **15**, 2435.
Mayneord, W. V. (1950). *Brit. J. Radiol.*, Suppl. No. 2, 142.
McMahon, H. B., Murphy, A. S., and Bates, M. (1947). *Amer. J. Path.*, **23**, 585.
Mole, R. H (1955). *J. Nat. Cancer Inst.*, **15**, 907.
Müller, B. (1954). *Acta Path et Microbiol. Scand.*, **35**, 549.
Moore, R A. (1945). *A Text-book of Pathology*. Philadelphia and London; W. B. Saunders
Moore, G E., Brackney, E. L., and Bock, F. G. (1953), *Proc. Soc. exp. Biol. Med.*, **82**, 643
Morris, H. P. (1955). *Advances in Cancer Research, III*. New York: Academic Press.
Mottram, J C. (1938). *Amer. J. Cancer*, **32**, 76.
Norris, W. P, Speckman, T. W., and Gustafson, P. F. (1955). *Amer. J. Roentgenol*, **73**, 785.
Owen, M , Jowsey, J , and Vaughan, J. (1955). *J. Bone Jt. Surg.*, **37B**, 324.
Passonneau, J. V., and Hamilton, K. (1951) *Argonne Nat. Lab.*, ANL-4625, 164.
Peller, S. (1939). *Human Biology*, **11**, 130
Pettit, V. D., Chamness, J. T., and Ackerman, L. V. (1954). *Cancer*, **7**, 149.
Raper, J. R., Henshaw, P. S., and Snider, R. S. (1951). In *Effects of External Beta Radiation*. (R. E. Zirkle.) New York; McGraw-Hill.
Raven, R. W., and Levison, V. B. (1954). *Lancet*, **2**, 683.
Ross, J. M. (1932). *J. Path. Bact.*, **35**, 899.
—— (1936). *Ibid.*, **43**, 267.
Rotblat, J., and Ward, G. B. (1953). *Nature*, **172**, 769.
Saunders, T. S., and Montgomery, H. (1938). *J. Amer. Med. Ass.*, **110**, 23.
Schürch, O. (1931a). *Z. Krebsforsch.*, **33**, 1.
—— (1931b). *Ibid.*, **33**, 35.
Sedgindse, G. A. (1933). *Z. Krebsforsch.*, **38**, 21.
Selbie, F. R. (1938) *Brit. J. exp. Path.*, **19**, 100.
Shubik, P., Goldfarb, A. R., Ritchie, A. C., and Lisco, H. (1953). *Nature*, **171**, 934.

Furth, J. and Butterworth, J. S. (1936). *Amer. J. Cancer*, 28, 66.
—— and Furth, O. B. (1936). *Ibid.*, 28, 54.
—— and Lorenz, E. (1954). *Radiation Biology*, Vol. 1, Part 2, Chap. 18. New York; McGraw Hill.
—— and Tullis, J. L. (1956). *Cancer Res.*, 16, 5.
—— and Upton, A. C. (1953). *Ann. Rev Nuclear Sci.*, 3, 303.
Gardner, W. U. (1950). *Proc. Soc. exp. Biol.*, 75, 434.
—— (1953). *Advances in Cancer Research*, Vol. 1, p. 173. New York; Academic Press.
—— (1955). *Cancer Res.*, 15, 109.
Ghormley, R. K. (1941). *Proc. Mayo Clin.*, 16, 69.
Glucksmann, A. (1945). *Cancer Res.*, 5, 385.
—— and Boag, J. W. (1951). *J. Path. Bact.*, 63, 176.
—— —— (1954). *Acta Radiol.*, Suppl. 116, 688.
Goebel, O., and Gérard, P. (1925). *Compt. rend. Soc. Biol.*, 93, 1537.
Goldberg, R. C., and Chaikoff, I. L. (1952). *Arch. Path.*, 53, 22.
Goolden, A. W. G. (1951). *Brit. med. J.*, 2, 1110.
Gorbman, A. (1949). *Proc. Soc. exp. Biol.*, 71, 237.
Gray, L. H. (1947). *Brit. J. Radiol.*, Suppl. No. 1.
Gricouroff, G. (1937). *Bull. Ass. franc. Cancer*, 26, 378.
—— (1943). *Mem. Acad. Chir. Paris*, 69, 529.
Grinspan, D., and Abulafih, J. (1955). *Cancer*, 8, 1047.
Grützmacher, K. T. (1942). *Strahlentherapie*, 72, 330.
Guimaraes, J. P., Lamerton, L. F., and Christensen, W. R. (1955). *Brit. J. Cancer*, 11, 253.
Hamilton, J. G. (1948). *Rev. Mod. Phys.*, 20, 718.
Harnett, W. L. (1952). "Survey of Cancer in London", *British Empire Cancer Campaign*.
Hatcher, C. H. (1945). *J. Bone Jt. Surg.*, 27, 179.
Henshaw, P. S, and Hawkins, J. W. (1944). *J. Nat. Cancer Inst.*, 4, 339.
—— Riley, E. F., and Stapleton, G. E. (1947). *Radiology*, 49, 349.
—— Snider, R. S., and Riley, E. F. (1949). *Ibid.*, 52, 401.
Hesse, O. (1911). *Fortschr. Rontgenstrahl.*, 17, 82.
Hoecker, F. E., and Roofe, P. G. (1951). *Radiology*, 56, 89.
Holthusen, H., and Englmann, K. (1931). *Strahlentherapie*, 42, 514.
Hultberg, S., and Larsson, L. E. (1950). *Acta Radiol.*, 33, 377.
Johansen, C. (1955) *Acta Path. Microbiol. Scand.*, Suppl. 105.
Jones, A. (1953). *Brit. J. Radiol.*, 26, 273.
Jonkhoff, A. R. (1928). *Z. Krebsforsch.*, 26, 32.
Kaplan, H. S. (1950). *J. Nat. Cancer Inst.*, 11, 125.
—— and Brown, M. B. (1951). *Ibid.*, 12, 427.
—— —— (1952). *Ibid.*, 13, 185.
—— —— (1954). *Science*, 119, 439
—— —— and Paull, J. (1953). *Cancer Res.*, 13, 677.
—— Moses, L. E., Brown, M. B., Nagareda, C. S., and Hirsch, B. B. (1955). *J. Nat. Cancer Inst.*, 15, 975.
—— and Murphy, E. D. (1949). *Ibid.*, 9, 407.
Koletsky, S. (1954). *Proc. Soc. exp. Biol.*, 86, 266
—— Bonte, F. J., and Friedell, H. L. (1950). *Cancer Res.*, 10, 129.
—— and Gustafson, G. E. (1955). *Ibid.*, 15, 100.
Krebs, C., Rask-Nielsen, H. C., and Wagner, A. (1930). *Acta Radiol.*, Suppl. X.
Lacassagne, A. (1933) *Compt. rend. Soc. Biol.*, 112, 562.
—— (1945a). *Actualités scientifiques et industrielles*, p. 975, Paris.
—— (1945b). *Ibid.*, p. 981, Paris.

Lacassagne, A. (1954). *Acta Union Intern. Contre le Cancer*, 10, 56.
——— and Vincent, R. (1929). *Compt. rend Soc. Biol.*, 100, 249.
Lange, R. D., Moloney, W. C., and Yamawaki, T. (1954). *Blood*, 9, 547.
Leddy, E. T., and Rigos, F. J. (1941). *Amer. J. Roentgenol.*, 45, 696.
Li, M. H., and Gardner, W. U. (1949). *Cancer Res*, 9, 35.
Lick, L., Kirschbaum, A., and Mixer, H. (1949) *Ibid.*, 9, 532.
Lisco, H., and Finkel, M P. (1949). *Fed. Proc.*, 8, 360.
Looney, W. B. (1955). *J. Bone Jt. Surg.*, 37A, 1169.
——— (1956). *Ibid.*, 38A, 160, 392
Lorenz, E. (1944). *J. Nat. Cancer Inst.*, 5, 1.
——— Heston, W. E., Deringer, M. K., and Eschenbrenner, A. B. (1946). *Ibid.*, 6, 349.
——— Eschenbrenner, A. B., and Deringer, M. K. (1947). *Radiology*, 49, 274.
——— (1950) *Amer. J. Roentgenol.*, 63, 176.
——— Eschenbrenner, A B., Heston, W. E., and Uphoff, D. (1951). *J. Nat. Cancer Inst.*, 11, 947.
——— Hollcroft, J. W., Miller, E., Congdon, C. C., and Schweistaal, R. (1955) *Ibid.*, 15, 1049
Ludin, M. (1934). *Acta Radiol.*, 15, 553.
March, H. C. (1944). *Radiology*, 4, 275.
——— (1950) *Amer. J. med. Sci.*, 220, 282.
Marie, P., Clunet, J., and Raulot-Lapointe, G. (1910). *Bull. Ass. franç. cancer*, 3, 404.
Marquès, P., Planel, H., and Hermous, G. (1952). *Ibid.*, 39, 214.
Martland, H S., Conlon, P., and Knef., J. P. (1925). *J. Amer. med. Ass.*, 85, 1769.
——— (1931). *Amer. J Cancer*, 15, 2435.
Mayneord, W. V. (1950). *Brit J. Radiol*, Suppl. No. 2, 142.
McMahon, H B., Murphy, A. S., and Bates, M. (1947). *Amer. J. Path.*, 23, 585.
Mole, R. H (1955). *J. Nat. Cancer Inst.*, 15, 907.
Müller, B (1954). *Acta Path et Microbiol. Scand.*, 35, 549.
Moore, R. A (1945). *A Text-book of Pathology*. Philadelphia and London; W. B. Saunders.
Moore, G. E., Brackney, E. L., and Bock, F. G. (1953). *Proc. Soc. exp. Biol. Med.*, 82, 643.
Morris, H P. (1955) *Advances in Cancer Research, III*. New York: Academic Press.
Mottram, J C (1938) *Amer J. Cancer*, 32, 76.
Norris, W. P., Speckman, T. W., and Gustafson, P. F. (1955). *Amer. J. Roentgenol*, 73, 785.
Owen, W., Jowsey, J., and Vaughan, J (1955). *J. Bone Jt. Surg.*, 37B, 324.
Passonneau, J V., and Hamilton, K. (1951). *Argonne Nat. Lab.*, ANL-4625, 164.
Peller, S. (1939). *Human Biology*, 11, 130.
Pettit, V. D., Chamness, J. T., and Ackerman, L. V. (1954). *Cancer*, 7, 149.
Raper, J. R., Henshaw, P. S., and Snider, R. S. (1951). In *Effects of External Beta Radiation*. (R. E. Zirkle.) New York; McGraw-Hill.
Raven, R. W., and Levison, V. B. (1954). *Lancet*, 2, 683.
Ross, J M (1932) *J. Path Bact.*, 35, 899.
——— (1936). *Ibid.*, 43, 267.
Rotblat, and Ward, G. B. (1953). *Nature*, 172, 769.
Saunders, T. S., and Montgomery, H. (1938). *J. Amer. Med. Ass.*, 110, 23.
Schürch, O. (1931a). *Z. Krebsforsch*, 33, 1.
——— (1931b) *Ibid*, 33, 35.
Sedginidse, G. A. (1933) *Z. Krebsforsch.*, 38, 21.
Selbie, F. R. (1938) *Brit. J. exp. Path.*, 19, 100.
Shubik, P., Goldfarb, A. R., Ritchie, A. C., and Lisco, H. (1953). *Nature*, 171, 934.

Sikl, H. (1950). *Acta Univ. Intern. Contra Cancrum*, 6, 1366.

Simpson, C. L., Hempelmann, L. H., and Fuller, L. M. (1955). *Radiology*, 64, 840.

Smith, F. W., Gardner, W. U., Li, M. H., and Kaplan, H. (1949). *Cancer Res.*, 9, 193.

Speert, H. (1952) *Cancer*, 5, 478.

Spiers, F. W. (1949). *Brit. J. Radiol.*, 22, 521.

—— (1951). *Ibid.*, 24, 365.

—— (1953). *Ibid*, 26, 296.

Spitz, S., and Higginbotham, N. L. (1951). *Cancer*, 4, 1107.

Sulzberger, M. B , Baer, R. L., and Borota, A. (1952). *Arch. Derm. Syph., Chicago*, 65, 639.

Teloh, H. A., Mason, M. L., and Wheelock, M. C. (1950). *Surg. Gyn. Obstet*, 90, 335.

Vogtlin, J., and Minder, W. (1952). *Radiol. Clin.*, 21, 96.

Walter, J. (1950). *Brit. med. J.*, 1, 273.

Winship, T. (1951). *Trans. Amer. Goiter Ass.*, 314.

Wintz, H., and Rump, W. (1931). *League of Nations Health Organisation Publications No. C.H.1054.*

STUDIES OF PLASMA VOLUME, RED CELL VOLUME AND TOTAL BLOOD VOLUME IN YOUNG GROWING RATS

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Previous determinations of blood volume in the rat, with the exception of a few early studies in which attempts were made to estimate red cell volume by complete exsanguination, have been based on dilution analysis following the injection of substances labelling either the plasma or the red cells. However, the estimation of total blood volume from measurements either of plasma volume or red cell volume together with haematocrit determinations on samples of peripheral blood takes no account of inequalities in the distribution of cells and plasma in different parts of the circulation, and may be grossly misleading. The discrepancies in early determinations may be judged from Table 1 which surveys values reported up to the present time. Total blood volume can be accurately found only by the summation of cell volume and plasma volume individually determined (Reeve, 1948).

Simultaneous measurements of red cell volume and plasma volume in large rats have recently been reported by Huang & Bondurant (1956). However, this investigation, like most studies of blood volume in the rat, has been aimed at the establishment of a value for blood volume per unit body weight without regard to the dependence of this value on the weight or age of the animal. Since the relationship between blood volume and body weight may not be linear, it is inadmissible to use a value for blood volume per unit body weight obtained experimentally in animals of one weight range in order to calculate blood volumes of animals in a different weight range. A number of investigators (Chisholm, 1911; Metcoff & Favour, 1944; Lippman, 1947; Wang & Hegsted, 1949a) have studied the relationship between blood volume and body weight in the rat, but their studies are based either on cell volume measurements alone or plasma volume measurements alone; their conclusions must therefore be accepted with reserve. The possibility of variations due to strain, sex and diet must also be admitted.

The study here described has been carried out in order to obtain reliable data on the dependence of plasma volume and red cell volume on body weight

TABLE 1. Values reported for blood volumes in the rat

Investigators	Method	Weight range (g)	Haematocrit	Red cell volume (ml./100 g)	Plasma volume (ml./100 g)	Total blood volume (ml./100 g)
Jolly & Stiel (1903)	Exsanguination	172–307	—	—	—	4–5
Chabrlom (1911)	Exsanguination	53–148	—	—	—	7.84–4.73 / 6.28 (mean) / $BV = 0.099\,W^{0.8}$
Berlin et al. (1949)	³⁵Fe or ³²P cells	150–320	45.8	2.16 ± 0.20	—	4.59 ± 0.37
Berlin et al. (1950)	³²P cells	61–73	45.0	2.87 (mean)	—	6.3 (mean)
		180–186	45.8	2.19 (mean)	—	4.81 (mean)
Sharpe et al. (1950)	³⁵Fe or ⁵⁹Fe cells	260–360	48.4	2.32 ± 0.16	—	4.95 ± 0.27
Montgomery (1951)	³²P cells	118–406	—	—	—	6.3 ± 0.7
Fryers (1952)	⁵⁹Fe cells	204–253	42.7	2.15 (mean)	—	4.97 (mean)
Costopoulos et al. (1954)	⁵⁹Fe cells	199–211	45.5	2.34 ± 0.05	—	5.4–7.9 / 6.13 (mean)
Scott & Barcroft (1924)	Gasometric dilution, CO	95–154	—	—	—	6.0–7.9 / 6.8 (mean)
Cartland & Koch (1928)	Dye dilution, vital red	101–262	—	—	—	6.9–7.9 / 7.4 (mean)
Went & Drinker (1929)	Dye dilution, vital red	189–321	—	—	—	5.78–6.92 / 6.38 (mean)
Orten et al. (1933)	Dye dilution, vital red	184–310	—	—	3.09–4.05 / 3.43 (mean)	4.1–5.3 / 4.3 (mean)
Griffith & Campbell (1937)	Dye dilution, vital red	Adult	—	—	—	—
Beckwith & Chantin (1941)	Dye dilution, Evans blue	3–4 months	—	—	4.08 ± 0.49	7.93 ± 0.85
Metcoff & Favour (1944)	Dye dilution, Evans blue	41–66	37.1	—	5.58 (mean)	8.90 (mean)
		73–89	35.8	—	6.49 (mean)	10.7 (mean)
		137–336	43.9	—	4.03 (mean)	7.18 (mean)
Lippman (1947)	Dye dilution, haemoglobin	48–308	—	—	$PV = 0.122\,W^{0.678}$	$BV = 0.1951\,W^{0.794}$
Wang & Hegsted (1949a, b)	Dye dilution, Evans blue	63–394	—	—	$PV = 0.182\,W^{0.571}$	$BV = 0.1911\,W^{0.811}$
Huang & Bondurant (1956)	Dye dilution, Evans blue and ³²P cells	230–550	46.1	2.63 (mean)	3.13 (mean)	5.75 (mean)

PV = plasma volume (ml./100 g); BV = total blood volume (ml./100 g); W = body weight (g)

in a pure-line strain of rat—the August hooded strain—originally obtained from Dr Anna Goldfeder of New York City Cancer Research Laboratory, and now used in many investigations at the Institute of Cancer Research. Plasma volume has been measured by dilution studies with Evans blue dye and also with ^{131}I-labelled human serum albumin, red cell volume by dilution studies with ^{59}Fe-labelled red cells obtained from donor animals.

METHODS

Normal male brown-hooded rats of the August strain were used throughout. They were maintained on Medical Research Council diet No. 41 and water *ad libitum*.

Preparation of donor animals for red cell volume measurements. Rats of weight approximately 150 g were injected intravenously with 10 μc ^{59}Fe as ferric chloride in 1% sodium citrate solution. After an interval of 14 days to allow maximum uptake of radioactive iron by the circulating red cells, by which time plasma ^{59}Fe content was negligible, blood was withdrawn by cardiac puncture into a heparinized syringe and injected without delay into recipient animals.

Preliminary studies of mixing conditions. Rats of different weights were anaesthetized with 0·12 ml./100 g of a 60 mg/ml. solution of sodium pentobarbitone (Nembutal, Abbott Laboratories Ltd.) injected intraperitoneally. The right carotid artery was cannulated with a fine polyethylene cannula attached to a 1 ml. syringe by a No. 20 needle. A small quantity of heparin solution was injected intravenously into a lateral tail vein and the needle used for this injection was left in position in the vein. Through this needle was then injected either:

(a) 0·2 ml. of a solution of 0·5% Evans blue (Imperial Chemical (Pharmaceuticals) Ltd.) in isotonic saline, or

(b) 0·2 ml. of a solution of ^{131}I-labelled human serum albumin (Abbott Laboratories Ltd.) in isotonic saline containing about 1 μc/ml. ^{131}I, or

(c) 0·2 ml. heparinized blood from a donor animal containing about 1 μc/ml. ^{59}Fe as labelled red cells.

At 1, 2, 5, 10, 15, 20, 30, 45, 60 min after injection blood samples of volume approximately 0·25 ml. were withdrawn via the carotid cannula and transferred to weighed sample tubes $\frac{3}{8}$ in. in diameter. The tubes were reweighed to give the weight of each sample by difference. The radioactive samples were assayed directly for ^{131}I or ^{59}Fe in a well-type scintillation counter (Anger, 1951) after which the blood was transferred to Wintrobe tubes for haematocrit determination. The Evans blue samples were transferred directly to Wintrobe tubes for haematocrit determination; after reading the haematocrits the supernatant plasma was pipetted off and dye concentration estimated by diluting a portion of plasma to 3 ml. and measuring optical density at 605 mμ against a diluted plasma blank in a Unicam SP 600 spectrophotometer. Haematocrit determinations were performed by centrifugation at 3000 rev/min for 30 min at a radius of 15 cm.

Measurement of plasma volume. Rats were lightly anaesthetized with ether. Either 0·2 ml. of a solution of 0·5% Evans blue in isotonic saline or 0·2 ml. of a solution of ^{131}I-labelled human serum albumin, in isotonic saline containing 1 μc/ml. ^{131}I, was injected intravenously into each animal by the lateral tail vein. Using the same syringe, further 0·2 ml. portions of the injected solution were measured into 10 ml. volumetric flasks and diluted to 10 ml for use as standards. Exactly 5 min after injection, a single 0·5 ml. sample of blood was withdrawn from each animal by cardiac puncture with a syringe moistened with heparin solution and transferred to a sample tube $\frac{3}{8}$ in. in diameter. 0·5 ml. portions of the standard dilution were similarly measured into other sample tubes. Samples and standards were assayed as already described

Measurement of red cell volume. Rats were lightly anaesthetized with ether. 0·2 ml. of heparinized blood containing 3 μc/ml. ^{59}Fe as labelled red cells was injected intravenously into each animal by the lateral tail vein. Using the same syringe, further 0·2 ml. portions of the injected blood were measured into 10 ml. volumetric flasks and diluted to 10 ml. with water for use as

TABLE 1. Values reported for blood volumes in the rat

Investigators	Method	Weight range (g)	Haematocrit	Red cell volume (ml./100 g)	Plasma volume (ml./100 g)	Total blood volume (ml./100 g)
Jolly & Susi (1905)	Exsanguination	172-307	—	—	—	4-5
Chiksola (1911)	Exsanguination	53-148	—	—	—	7·84 – 4·73; 6·28 (mean); $BV = 0.090 W^{0.80}$
Berlin et al. (1949)	^{55}Fe or ^{32}P cells	150-320	45·8	2·16±0·20	—	4·59±0·57
Berlin et al. (1950)	^{32}P cells	61-73	45·0	2·87 (mean)	—	6·3 (mean)
		180-186	45·8	2·19 (mean)	—	4·81 (mean)
Sharpe et al. (1950)	^{55}Fe or ^{59}Fe cells	260-360	48·4	2·32±0·16	—	4·95±0·27
Montgomery (1951)	^{32}P cells	118-406	—	—	—	6·3 ±0·7
Fryers (1952)	^{59}Fe cells	204-253	42·7	2·15 (mean)	—	4·97 (mean)
Contopoulos et al. (1954)	^{59}Fe cells	199-211	46·5	2·34±0·05	—	—
Scott & Barcroft (1924)	Gasometric dilution, CO	95-154	—	—	—	5·4-7·9; 6·13 (mean)
Cartland & Koch (1928)	Dye dilution, vital red	101-262	—	—	—	6·0-7·9
West & Dinker (1929)	Dye dilution, vital red	180-321	—	—	—	6·9-7·9
Orten et al. (1933)	Dye dilution, vital red	184-310	—	—	—	7·4 (mean)
Griffith & Campbell (1937)	Dye dilution, vital red	Adult	—	—	—	5·78-6·92; 6·38 (mean)
Beckwith & Chanutin (1941)	Dye dilution, Evans blue	3-4 months	—	—	4·08±0·49	4·1-5·3; 4·3 (mean)
Metcoff & Favour (1944)	Dye dilution, Evans blue	41-68	37·1	—	5·58 (mean)	7·08±0·85
		73-89	35·8	—	6·49 (mean)	8·90 (mean)
		137-330	43·9	—	4·03 (mean)	10·7 (mean); 7·18 (mean)
Lippman (1947)	Dye dilution, haemoglobin	48-308	—	—	—	—
Wang & Hegsted (1949 a, b)	Dye dilution, Evans blue	63-394	—	—	$PV = 0.122 W^{0.71}$	$BV = 0.105 W^{0.74}$
Huang & Bondurant (1956)	Dye dilution, Evans blue and ^{32}P cells	230-550	46·1	2·63 (mean)	3·13 (mean)	5·75 (mean)

PV = plasma volume (ml/100 g); BV = total blood volume (ml./100 g); W = body weight (g).

Fig. 2. Mixing curves for Evans blue injected intravenously into August rats. ●, Observed concentration of dye in plasma; ○, dye concentration corrected for amount removed in previous samples.
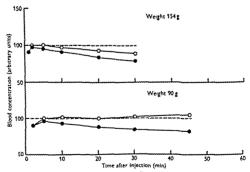
Fig. 3. Mixing curves for ^{131}I-labelled human serum albumin injected intravenously into August rats. ●, Observed ^{131}I concentration in circulating blood; ○, ^{131}I concentration corrected for activity removed in previous samples.

standards. Exactly 10 min after injection a single 0·5 ml. sample of blood was withdrawn from each animal by cardiac puncture with a syringe moistened with heparin solution and transferred to a sample tube ¾ in. in diameter. 0·5 ml. portions of the standard dilution were similarly measured into other sample tubes Samples and standards were then assayed as already described.
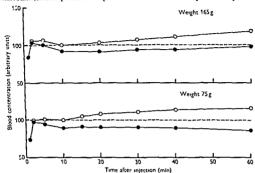
Fig. 1. Mixing curves for ^{59}Fe-labelled red cells injected intravenously into August rats. ●, Observed ^{59}Fe concentration in circulating blood; ○, ^{59}Fe concentration corrected for activity removed in previous samples.

RESULTS

Preliminary studies of mixing conditions

In order to study the mixing of the injected materials in the circulating blood and to establish a valid sampling technique for routine blood volume determinations, preliminary studies were made using animals with one carotid artery cannulated for serial blood sampling. Injection was performed by tail vein and 0·2 ml. blood samples withdrawn for analysis at intervals during the first hour after injection. Fig. 1 shows typical results for two animals of different weights injected with ^{59}Fe-labelled red cells. Figs. 2 and 3 show similar results for animals injected with Evans blue and ^{131}J-labelled human serum albumin respectively. The experimental values for concentration of the injected material in the circulating blood are shown both uncorrected and corrected for material removed from the circulation in previous samples. This correction has been performed using the relationship

$$\text{Corrected concn.} = \text{Observed concn.} \times \frac{T_I}{T_I - T_R}.$$

where T_I = total amount injected, T_R = total amount removed in previous samples.

injection with Evans blue and ^{131}I-labelled albumin or 10 min after injection with ^{59}Fe-labelled red cells, and analysed as described.

Plasma and red cell volumes were calculated using the following relationships:

$$PV = \frac{10 \times EB_S \times 100}{EB_P \times W},$$

where PV = plasma vol. (ml./100 g),

EB_S = Evans blue concn. in standard,

EB_P = Evans blue concn. in plasma,

W = body wt. (g);

or

$$PV = \frac{10 \times I_S \times Pt \times 100}{I_B \times W},$$

where I_S = ^{131}I counting rate from 0·5 ml. standard,

I_B = ^{131}I counting rate from 0·5 ml. blood sample,

Pt = corrected % plasma in blood sample;

$$RCV = \frac{\left[\dfrac{10 \times Fe_S \times Ht}{Fe_B} - RC_I\right] \times 100}{W},$$

where RCV = red cell vol. (ml./100 g),

Fe_S = ^{59}Fe counting rate from 0·5 ml. standard,

Fe_B = ^{59}Fe counting rate from 0·5 ml. blood sample,

Ht = corrected % red cells in blood sample,

RC_I = volume of red cells injected (ml.).

Haematocrit readings were corrected for trapped plasma assuming a value of 4% of plasma in the total packed cell column (Huang & Bondurant, 1956). Red cell volumes were corrected for the total volume of labelled red cells injected; this was usually 0·1 ml. No such correction was thought necessary in plasma volume measurements where homoeostasis may be expected to maintain constancy of blood volume after injection. Results are shown in Fig. 4.

Measurements of plasma volume with ^{131}I-labelled human serum albumin usually showed good agreement with those made by the Evans blue method. However, two batches of ^{131}I-labelled albumin gave anomalous results; simultaneous determinations of plasma volume by the ^{131}I-labelled albumin and dye methods in which samples from these batches were injected mixed with Evans blue showed over-estimation of plasma volume by the ^{131}I-labelled albumin, whilst studies of the mixing curves showed a rapid initial loss of ^{131}I from the circulation amounting to some 20% of the injected material. For this reason

The uncorrected mixing curves following the injection of labelled red cells and labelled plasma are similar in form. The low concentration of injected material in the first sample withdrawn in each experiment is due to dead space blood in the cannula. After the second sample, the blood concentration falls. This fall is at first mainly attributable to the mixing of the injected material in the circulation, but may later be due in part to the effects of repeated sampling and in part to the loss of injected material to extravascular compartments.

The results for ^{59}Fe-labelled cells are of especial interest since no significant loss of labelled cells from the circulation is to be expected in the duration of the experiment. Inspection of Fig. 1 shows that the initial mixing phase is complete after 10 min. Thereafter the uncorrected curves of blood ^{59}Fe concentration fall only slightly despite repeated sampling, showing that the homoeostatic mechanisms maintaining the constancy of blood volume are not able to compensate for the loss of successive samples. The corrected curves therefore show an apparent rise in concentration, reflecting a reduced blood volume. These effects are particularly clear in the smaller animal studied, in which the blood volume has fallen significantly after 15 min. In the larger animal blood volume is approximately constant up to 20 min after injection.

Consideration of Fig. 1 suggests that a sampling technique in which a single sample is taken at the end of the initial mixing period 10 min after injection is satisfactory for the estimation of blood volume with ^{59}Fe-labelled red cells in the rat.

The results for Evans blue and for ^{131}I-labelled albumin are similar save that the uncorrected mixing curves show a steady fall after the initial mixing phase due to the removal of injected material from the circulation. If the blood volume is assumed constant in the larger animals up to 20 min after injection, the rate of loss of Evans blue and ^{131}I-labelled albumin may be estimated from Figs. 2 and 3 to be 22 %/hr and 25 %/hr respectively. To correct for loss during mixing, the mixing curve should properly be extrapolated back to zero time. From Figs. 2 and 3 it is however considered that a single sample withdrawn 5 min after injection gives a sufficiently accurate estimate of the extrapolated blood concentration of Evans blue or ^{131}I-labelled albumin at zero time in the rat, loss of injected material during the first 5 min being approximately compensated by the slightly incomplete mixing after this time.

Measurements of plasma volume and red cell volume

Following these initial studies, plasma volume and red cell volume measurements were performed on a large number of rats. Animals were injected with Evans blue, ^{131}I-labelled human serum albumin or ^{59}Fe-labelled red cells by tail vein. Single blood samples were taken by cardiac puncture 5 min after

determination of plasma volume and red cell volume in large rats by Huang & Bondurant (1956) using a double sampling technique. It is questionable, however, whether such a procedure is valid in small animals where the volume of each sample withdrawn is a significant fraction of the total blood volume, since the concentration of injected material in later samples may be decreased by virtue of the amount removed in earlier samples. If it be assumed that the total blood volume and haematocrit remain constant, then it is possible to

TABLE 2. Plasma volumes, red cell volumes and total blood volumes in male August rats

Weight range (g)	Plasma volume (ml./100 g)	Red cell volume (ml./100 g)	Total blood volume (ml./100 g)	Cardiac haematocrit (% red cells) A	Mean body haematocrit (% red cells) B	$\dfrac{B}{A}$
26–50	5·38 ± 0·26	2·21 ± 0·14	7·59 ± 0·27	36·5	29·1	0·80
51–75	4·76 ± 0·31	2·34 ± 0·10	7·10 ± 0·30	39·1	33·0	0·84
76–100	4·66 ± 0·22	2·38 ± 0·13	7·04 ± 0·24	41·8	33·8	0·81
101–125	4·55 ± 0·25	2·47 ± 0·14	7·02 ± 0·25	42·6	35·2	0·83
126–150	4·13 ± 0·30	2·54 ± 0·10	6·67 ± 0·28	45·5	38·1	0·83
151–175	3·86 ± 0·20	2·45 ± 0·07	6·31 ± 0·19	45·8	38·8	0·84
176–200	3·29 ± 0·19	2·39 ± 0·18	5·68 ± 0·24	46·7	42·7	0·90
201–225	3·02 ± 0·31	2·25 ± 0·22	5·27 ± 0·34	48·5	42·7	0·88
226–250	3·03 ± 0·24	2·07 ± 0·22	5·10 ± 0·26	48·5	40·7	0·84

The errors quoted are fiducial limits for $P = 0.05$.

Fig. 5. Mean values of total blood volume, plasma volume, red cell volume and haematocrit in August rats. The errors shown are fiducial limits for $P = 0.05$.

less confidence is placed in the results obtained with ^{131}I-labelled human serum albumin than those with Evans blue, and the former have not been included in the detailed analysis of data.
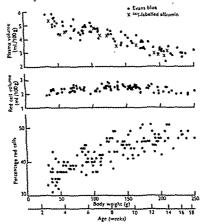
Fig. 4. Plasma volume, red cell volume and haematocrit values in August rats.

Table 2 and Fig. 5 show the results grouped according to body weight. Total blood volume was calculated as the sum of plasma volume and red cell volume, the small contribution (1–2%) of the white cells being neglected. The final column of Table 2 gives the ratio of mean body haematocrit, calculated from the experimentally derived values for red cell volume and total blood volume to the haematocrit measured on a sample of cardiac blood; both haematocrits are expressed as the percentage of red cells in total blood excluding white cells.

DISCUSSION

A fundamental difficulty in the measurement of blood volume in small animals by dilution methods arises from the time required for uniform mixing of the injected material and the loss of injected material to extra-vascular compartments during the mixing phase. In large species this difficulty may be overcome by serial blood sampling and extrapolation of the blood concentration curve back to the time of injection. This method has been applied to the

determination of plasma volume and red cell volume in large rats by Huang & Bondurant (1956) using a double sampling technique. It is questionable, however, whether such a procedure is valid in small animals where the volume of each sample withdrawn is a significant fraction of the total blood volume, since the concentration of injected material in later samples may be decreased by virtue of the amount removed in earlier samples. If it be assumed that the total blood volume and haematocrit remain constant, then it is possible to

TABLE 2. *Plasma volumes, red cell volumes and total blood volumes in male August rats*

Weight range (g)	Plasma volume (ml./100 g)	Red cell volume (ml./100 g)	Total blood volume (ml./100 g)	Cardiac haematocrit (% red cells) A	Mean body haematocrit (% red cells) B	$\frac{B}{A}$
26–50	5·38±0·26	2·21±0·14	7·59±0·27	36·5	29·1	0·80
51–75	4·76±0·31	2·34±0·10	7·10±0·30	39·1	33·0	0·84
76–100	4·66±0·22	2·38±0·13	7·04±0·24	41·8	33·8	0·81
101–125	4·55±0·25	2·47±0·14	7·02±0·25	42·6	35·2	0·83
126–150	4·13±0·30	2·54±0·10	6·67±0·28	45·5	38·1	0·85
151–175	3·86±0·20	2·45±0·07	6·31±0·19	45·8	38·8	0·84
176–200	3·29±0·19	2·39±0·18	5·68±0·24	46·7	42·7	0·90
201–225	3·02±0·31	2·25±0·22	5·27±0·34	48·5	42·7	0·88
226–250	3·03±0·24	2·07±0·22	5·10±0·26	48·5	40·7	0·84

The errors quoted are fiducial limits for $P = 0.05$.
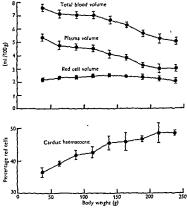
Fig. 5. Mean values of total blood volume, plasma volume, red cell volume and haematocrit in August rats. The errors shown are fiducial limits for $P = 0.05$.

correct the measured concentration of later samples for the amount of injected material previously removed. If, on the other hand, the blood volume is not maintained but decreases in proportion to the amount of blood removed, then no correction is necessary. In practice, as is shown by Figs. 1–3, homoeostatic mechanisms operate, but the blood volume may still fall during repeated sampling, particularly in small animals. As a result, a systematic under-estimation of the blood volume may result if the uncorrected data are extra-polated, while correction of the data based on the assumption that the blood volume remains constant may lead to an overestimation. It is therefore considered that a single sampling technique is to be preferred; the errors that result from such a technique are in any case small compared with the variation arising from biological and other causes. The interval between injection and sampling must depend on the nature of the injected material; intervals have been quoted ranging from 2 min for injections of haemoglobin solution (Lippman, 1947) to 2 hr for ^{59}Fe-labelled red cells. The studies reported here indicate that a mixing time of 5 min is satisfactory for Evans blue or ^{131}I-labelled albumin, but that 10 min should be allowed for ^{59}Fe-labelled red cells.

The measurements of plasma volume with ^{131}I-labelled albumin reported here are not sufficient in number for a detailed comparison of this with the Evans blue technique to be made, though no serious discrepancy between the two sets of results can be observed. Huang & Bondurant (1956) found good agreement between simultaneous measurements of plasma volume with Evans blue and ^{131}I-labelled albumin mixed before injection. By contrast, Wish, Furth & Storey (1950), in simultaneous measurements in mice, found that the plasma volume estimated with Evans blue was consistently higher by a factor 1·2 than that estimated with ^{131}I-labelled protein; they attributed the dis-crepancy to a rapid early loss of dye from the circulation, and corrected their Evans blue data by this factor. A similar rapid initial loss of Evans blue was reported by Cruickshank & Whitfield (1945) in the cat. No such fall in the Evans blue concentration of the plasma can however be observed in the mixing curves of Fig. 3, though it may be mentioned that the concentrations used were considerably higher than those used by the latter workers.

Freinkel, Schreiner, Athens, Hiatt & Breese (1954) found that the success of simultaneous determinations in man depended on the ratio of the amounts of Evans blue and labelled albumin injected; they postulated a reaction between the two substances leading to a rapid clearance of the latter under certain conditions. Similar effects have not been observed in the present studies. The anomalous results obtained with certain batches of labelled albumin in these studies may have been due to incomplete protein binding of the isotopic label, to partial denaturation of the protein during the labelling process or to rapid clearance of foreign protein from the circulation. No attempt was made to identify the nature of the observed effects. It is, however, recommended in

view of the results reported that ^{131}I-labelled albumin and Evans blue should only be used for blood volume studies in rats if detailed mixing curves have been followed and show no rapid early loss of the labelling substance from the circulation.

The results of the investigations show that it is incorrect to apply a single value for blood volume per unit weight to rats of different weights. The total blood volume falls steadily with increase in weight over the weight range studied, but this fall is not paralleled by a similar fall in red cell volume; on the contrary, the red cell volume is seen to be approximately constant over the weight range 40–250 g falling only slightly at the upper and lower ends of this range. The anaemia of the young growing rat in this weight range is thus seen to be mainly artifactual and to arise from the increased plasma volume per unit body weight in young animals. The slight fall in red cell volume observed in animals of body weight less than 100 g may reflect some degree of neo-natal anaemia. The fall observed at weights greater than 200 g may be related to the increased proportion of adipose tissue in adult rats; similar findings in man have been reported (Huff & Feller, 1956). The red cell volume data reported here are in reasonable agreement with those of Berlin, Huff, Van Dyke & Hennessy (1949), Sharpe, Culbreth & Klein (1950), Fryers (1952) and Contopoulos, Ellis, Simpson, Lawrence & Evans (1954) but show a somewhat lower value in small animals than that reported by Berlin, Van Dyke, Siri & Williams (1950). The value of 2·87 ml./100 g in rats of weight 61–73 g quoted by the latter authors is however based only on six observations.

A recent study of red cell volume and plasma volume as a function of age in the rat has been made by Garcia (1956) on the basis of red cell volume measurements with ^{59}Fe-labelled cells and haematocrit determinations. Since the latter were made not on cardiac or venous blood, but on blood obtained by exsanguinating the animals as completely as possible, the plasma volumes reported are probably not greatly in error despite the fact that they are based on measurements with labelled red cells. Both the red cell and plasma volumes reported are in reasonable agreement with those of Table 2, save for a marked fall in red cell volume in rats of weight less than 100 g, which was not observed in the present investigation. This may represent differences due to strain or diet; such differences are also suggested by the published growth data.

Metcoff & Favour (1944), Lippman (1947) and Wang & Hegsted (1949a) have made measurements of plasma volume by dye dilution methods in animals of different weights. These workers attempted to demonstrate a linear regression between the logarithms of body weight and plasma volume or total blood volume, representing a power law relationship between blood volume and body weight. Thus, Lippman (1947) found his data to fit the relationship:

$$\text{Plasma volume} = 0.122 \ (\text{body weight})^{0.778}.$$

correct the measured concentration of later samples for the amount of injected material previously removed. If, on the other hand, the blood volume is not maintained but decreases in proportion to the amount of blood removed, then no correction is necessary. In practice, as is shown by Figs. 1–3, homoeostatic mechanisms operate, but the blood volume may still fall during repeated sampling, particularly in small animals. As a result, a systematic underestimation of the blood volume may result if the uncorrected data are extrapolated, while correction of the data based on the assumption that the blood volume remains constant may lead to an overestimation. It is therefore considered that a single sampling technique is to be preferred; the errors that result from such a technique are in any case small compared with the variation arising from biological and other causes. The interval between injection and sampling must depend on the nature of the injected material; intervals have been quoted ranging from 2 min for injections of haemoglobin solution (Lippman, 1947) to 2 hr for ^{59}Fe-labelled red cells. The studies reported here indicate that a mixing time of 5 min is satisfactory for Evans blue or ^{131}I-labelled albumin, but that 10 min should be allowed for ^{59}Fe-labelled red cells.

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of 0·88 in mice, whilst Armin, Grant, Pels & Reeve (1952) obtained a value of 0·85 in albino rabbits. In dogs, Gibson, Peacock, Seligman & Sack (1946) found a ratio of 0·91, but Reeve, Gregersen, Allen & Sear (1953) found 0·988, this being reduced to 0·899 after splenectomy. In man, the ratio has been quoted as 0·88 by Gregersen (1951) and as 0·91 by Chaplin, Mollison & Vetter (1953). Earlier reported values are reviewed by Reeve (1948).

The data of Table 2 suggest that the haematocrit ratio increases slightly with body weight over the range studied. Detailed analysis of the data shows
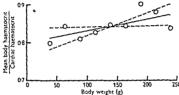
Fig. 6 Variation of haematocrit ratio with body weight in August rats The regression line has the equation

Haematocrit ratio = 0·80 + 0·00031 body weight.

The dotted lines show fiducial limits to the slope corresponding to $P = 0.05$.

that the linear regression of the value of the ratio on body weight in fact has a slope which is just significantly greater than zero at the 5% level of probability (Fig. 6) The value of the ratio determined from the regression line ranges from 0·81 at 37·5 g body weight to 0·87 at 237·5 g body weight. This finding provides further evidence that the changes observed in blood volume per unit body weight are related to the haemodynamic requirements of small blood vessels. In small animals, the proportion of the total blood volume contained in smaller vessels is increased, and the haematocrit ratio is thereby reduced.

SUMMARY

1 Measurements were made of plasma volume, red cell volume and cardiac haematocrit in male rats of the 'August' strain of weight range 31–250 g. Plasma volume was measured by dilution analysis with Evans blue dye and also with ^{131}I-labelled human serum albumin. Red cell volume was measured by dilution analysis with ^{59}Fe-labelled red cells.

2. Preliminary studies of mixing conditions indicated that satisfactory estimation of both plasma and red cell volume is possible by single sampling techniques, a sample of cardiac blood being taken 5 min after injection for the plasma volume measurements and 10 min after injection for the red cell volume measurements.

On the other hand, both Metcoff & Favour (1944) and Wang & Hegsted (1949a)
found that the relation between blood volume and body weight changed at
puberty and that their data could not adequately be represented by a single
regression line. The plasma volume data reported here are in reasonable
agreement with the values quoted by Lippman (1947) and Wang & Hegsted
(1949a). Since, however, there seems no adequate physiological basis for a
simple power relationship between either plasma volume or total blood volume
and body weight or body surface area, attempts to express experimental data
in terms of such a relationship appear unprofitable, and indeed misleading.
Moreover, because the workers cited have studied whole blood volume in terms
of plasma volume measurements alone their results fail to demonstrate the
relative constancy of red cell volume per unit body weight during growth.

It seems probable that the increased plasma volume of young rats is related
to differences in distribution of cells and plasma in vessels of different size,
and in particular to the haemodynamic requirements for a marginal layer of
plasma in small vessels (Krogh, 1929; Gibson, Seligman, Peacock, Aub, Fine &
Evans, 1946). If this explanation is correct, then it is to be expected that the
pattern observed in these studies will be found to be a general one, and that
the total blood volume per unit body weight will fall and mean body haemato-
crit will rise with increase in body weight in any chosen species, particularly
in small animals. Confirmation of this hypothesis must await detailed investi-
gations of plasma and red cell volume in small species, which are at present
lacking. Ancill (1956) reports a constant value of 7·20 ml./100 g body weight
for total blood volume in guinea-pigs in the weight range 250–750 g on the
basis of plasma volume studies with Evans blue, but gives no data relating to
smaller animals. Wish et al. (1950) describe simultaneous measurements of red
cell volume with ^{32}P-labelled red cells and plasma volume with Evans blue and
^{131}I-labelled albumin in mice, quoting a mean red cell volume of 2·99 ml./100 g
and a plasma volume of 5·06 ml./100 g in normal animals, but give no infor-
mation regarding the weights of their animals.

Unequal distribution of cells and plasma in vessels of different size is also
indicated by the values quoted in Table 2 for the ratio of mean body haemato-
crit to cardiac haematocrit, which shows a mean value of 0·84 ± 0·025 over the
entire weight range. This is in general agreement with measurements of the
ratio in other species but not with the figure of 0·987, reduced to 0·942 after
splenectomy, reported by Huang & Bondurant (1956) in rats of 230–550 g
weight. It may be that the high ratios reported by these workers arise, as they
suggest, from a rapid loss of unbound ^{32}P from the circulation after injection,
leading to an over-estimation of red cell volume. Values for the haematocrit
ratio in other species have been deduced from simultaneous estimation of
plasma volume and red cell volume by a number of workers, values for man
and dog are reviewed by Reeve (1948). Wish et al. (1950) found a mean value

GIBSON, J. G., SELIGMAN, A. M., PEACOCK, W. C., AUB, J. C., FINE, J. & EVANS, R. D. (1946). The distribution of red cells and plasma in large and minute vessels of the normal dog determined by radioactive isotopes of iron and iodine. *J. clin. Invest.* **25**, 848–857.

GREGERSEN, M. I. (1951). Blood volume. *Annu. Rev. Physiol.* **13**, 397–407.

GRIFFITH, J. Q. & CAMPBELL, R (1937). A method for determining blood volume in rats. *Proc. Soc. exp. Biol., N.Y.*, **36**, 38–40.

HUANG, K. C. & BONDURANT, J. H. (1956). Simultaneous estimation of plasma volume, red cell volume and thiocyanate space in unanaesthetized normal and splenectomized rats. *Amer. J. Physiol.* **185**, 441–445.

HUFF, R. L. & FELLER, D. D. (1956). Relation of circulating red cell volume to body density and obesity. *J. clin. Invest.* **35**, 1–10.

JOLLY, J. & STINI, J. (1905). Masse totale du sang chez le rat blanc. *C.R. Soc. Biol., Paris*, **58**, 835–837.

KROGH, A. (1929). *The Anatomy and Physiology of the Capillaries*, pp. 28–30. New Haven: Yale University Press.

LIPPMAN, R. W. (1947). Blood, plasma and 'drawn blood' volumes in the rat. *Proc. Soc. exp. Biol., N.Y.*, **66**, 188–191.

METCOFF, J. & FAVOUR, C. B. (1944). Determination of blood and plasma volume partitions in the growing rat. *Amer. J. Physiol.* **141**, 695–706.

MONTGOMERY, P. O. (1951). A method for determining blood volume of the rat using radioactive phosphorus. *Proc. Soc. exp. Biol., N.Y.*, **77**, 445–447.

ORTEN, J. M, UNDERHILL, F. A., MUSGRAVE, E. R. & LEWIS, R. P. (1933). Blood volume studies in cobalt polycythemia. *J. biol. Chem.* **99**, 457–463.

REEVE, E. B. (1948). Methods of estimating plasma and total red cell volume *Nutr. Abstr. Rev.* **17**, 811–834.

REEVE, E B, GREGERSEN, M. I, ALLEN, T. H. & SEAR, H. (1953). Distribution of cells and plasma in the normal and splenectomized dog and its influence on blood volume estimations with P32 and T 1824. *Amer. J. Physiol.* **175**, 195–203.

SCOTT, J. M. D. & BARCROFT, J. (1924). The blood volume and the total amount of haemoglobin in anaemic rats. *Biochem J.* **18**, 1–8.

SHARPE, L. M., CULBRETH, G C. & KLEIN, J. R (1950). Blood and packed cell volume of the adult rat as measured by tagged cells. *Proc. Soc. exp. Biol , N.Y.*, **74**, 681–685.

WANG, C. F. & HEGSTED, D. M. (1949a). Normal blood volume, plasma volume and thiocyanate space in rats and their relation to body weight *Amer. J. Physiol.* **156**, 218–226

WANG, C F. & HEGSTED, D. M (1949b). Determination of blood and plasma volumes, thiocyanate space and bromsulfalein clearance in rats. *Amer. J. Physiol.* **156**, 227–232.

WENT, S. & DRINKER, C. K. (1929). A micromethod for determination of the absolute blood volume, with data upon the blood volume of the guinea pig, white rat, rabbit and cat *Amer. J Physiol.* **88**, 468–478.

WISH, L., FURTH, J. & STOREY, R. H. (1950). Direct determination of plasma, cell and organ blood volumes in normal and hypervolemic mice. *Proc. Soc. exp. Biol., N.Y.*, **74**, 644–648.

3. Detailed values of plasma volume, red cell volume and haematocrit of animals in different weight ranges are presented. These data demonstrate that it is invalid to use a single value for blood volume per unit body weight to calculate blood volumes of animals of different weights. Red cell volume per unit body weight is approximately constant over the weight range studied but plasma volume per unit body weight falls continuously with increase in weight. It is suggested that the increased plasma volume observed in small animals is related to haemodynamic factors governing blood flow in small vessels.

4. The value of the ratio of calculated mean body haematocrit to the observed cardiac blood haematocrit increases slightly with increase in body weight, rising from 0·81 at body weight 37·5 g to 0·87 at body weight 237·5 g, with a mean value of 0·84 ± 0·025 over the entire weight range.

REFERENCES

ANCILL, R. J. (1956) The blood volume of the normal guinea-pig. *J. Physol.* **132**, 469–475.

ANGER, H. O. (1951). Scintillation counters for radioactive sample measurement. *Rec. sci. Instrum* **22**, 912–914

ARMIN, J., GRANT, R. T., PELS, H. & REEVE, E. B. (1952). The plasma, cell and blood volumes of albino rabbits as estimated by the dye (T1824) and ³²P marked cell methods. *J. Physiol* **116**, 59–73.

BECKWITH, J. R. & CHANUTIS, A. (1941) Blood volumes in hypertensive partially nephrectomised rats. *Proc. Soc. exp. Biol., N.Y.*, **46**, 66–69

BERLIN, N. I., HUFF, R. L., VAN DYKE, D. C. & HENNESSY, T. G. (1949) The blood volume of the adult rat, as determined by Fe⁵⁹ and P³² labelled red cells. *Proc. Soc. exp. Biol., N.Y.*, **71**, 176–178

BERLIN, N. I., VAN DYKE, D. C., SIRI, W. E. & WILLIAMS, C. P. (1950). The effect of hypophysectomy on the total circulating red cell volume of the rat *Endocrinology*, **47**, 429–435.

CARTLAND, G. F. & KOCH, F. C. (1928) A micro-modification of the Keith-Rowntree plasma-dye method for the estimation of blood volume in the rat. *Amer J Physiol* **85**, 540–545.

CHAPLIN, H., MOLLISON, P. L. & VETTER, H. (1953) The body, venous haematocrit ratio: its constancy over a wide haematocrit range. *J clin Invest.* **32**, 1309–1316

CHISHOLM, R. A. (1941). On the size and growth of the blood in tame rats. *Quart J. exp. Physiol.* **4**, 207–229

CONTOPOULOS, A. N., ELLIS, S., SIMPSON, M. E., LAWRENCE, J. H. & EVANS, H. M. (1954). Production of polycythemia in hypophysectomised rats by pituitary erythropoietic factor. *Endocrinology*, **55**, 808–812

CRUICKSHANK, E. W. H. & WHITFIELD, I. C (1945) The behaviour of T1824 (Evans blue) in the circulating blood and a modified method for the estimation of plasma volume. *J. Physiol.* **104**, 52–59

FREEMAN, S., SCHREINER, G. E., ATHENS, J. W., HIATT, C. W. & BARKER, S. (1954) Artifactual differences in the distribution of T1824 and P³² labelled albumin resulting from mixing prior to administration *J Lab clin Med* **43**, 212–220.

FREEMAN, G. R. (1952) Effects of decreased atmospheric pressure on blood volume of rats. *Amer. J Physiol* **171**, 474–478

GARCIA, J. F. (1954) Studies on erythropoiesis as a function of age in the normal male rat *University of Cal Ernst Radiation Laboratory Report No. UCRL-2516.*

GIBSON, J. G., PEACOCK, W. C., SELIGMAN, A. M. & SACK, T. (1946) Circulating red cell volume measured simultaneously by the radioactive iron and dye methods. *J. clin. Invest* **25**, 838–847

to a large extent fulfil these conditions: thulium-170 and xenon-133.

Thulium-170 is prepared from normal non-radioactive thulium-169 by placing it in the strongest available neutron flux of an atomic pile. The radioactive isotope is formed by neutron capture and proceeds to decay with a half-life of 127 days.

The decay scheme is complex (Mayneord, 1952; Mayneord and Ireland, 1956; Lidén and Starfelt, 1953) but, briefly, is by β-emission by two routes, one to stable ytterbium and the other to an excited state of ytterbium which emits γ-radiation of 84 kV and also its own characteristic x-radiation of 52 kV and 59.4 kV by a process known as internal conversion. In addition, there is a continuous broad band of bremsstrahlung radiation with a mean energy of about 150 kV. This is caused by the deceleration of the β-rays in the thulium, the mechanism being very similar to the production of x-rays in a normal x-ray tube.

Xenon-133, a gas, is a fission by-product of pile operation, it occurs in large quantities and has to be extracted by chemical and physical processes (Richards, 1955).

The dec   emits a γ-r   31 kV and

For radiographic purposes it is well understood that for the highest definition we must have as near a point source as possible; this necessarily implies a high concentration of activity if the times of exposure are not to be excessive.

In the case of thulium-170 it has been possible to irradiate a disc of sintered thulium oxide, 2 mm. in diameter and 2 mm. thick, and reach an activity of several curies with dose-rates of up to 3 mr/minute at 30 cm. To prevent leakage of the active material and to absorb the unwanted β-rays the active disc is sealed into a light alloy capsule which is itself sealed into a second capsule, the total thickness of alloy being about 2.25 mm.

Xenon-133 radioactive sources are prepared by absorption of the gas on to charcoal at the temperature of liquid nitrogen (Richards, 1955); on reaching normal room temperatures it is found that most of the gas remains strongly absorbed.

Activities of one to two curies have been concentrated in charcoal grains of about 1.5 mm. in diameter. Sources of up to 13 curies have been made by absorbing the gas in a charcoal rod 1 mm. in diameter by 5 mm. long. The rod is sealed in a glass capillary tube and is viewed end-on in order to achieve small focal size. Such sources give dose-rates of up to 75 mr/minute at 30 cm.

APPARATUS FOR CLINICAL USE

For practical purposes the primary sources described above must be shielded and a suitable shutter mechanism arranged so that exposures can be made when required. The shielding must be adequate, so that when the shutter is closed the source is safe to handle; due to the low energy of the sources this is easy to attain. For both the thulium-170 and xenon-133 sources, of the above orders of magnitude, 0.6 cm. of lead is sufficient to reduce the dose-rate to less than the maximum permissible of 2 mr/hour at one foot from the source. Theoretically the best absorber is gold, especially of radiations in the region of 80 to 100 kV (Mayneord, 1952; Mayneord and

RADIOGRAPHY
WITH RADIOACTIVE ISOTOPES

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THE naturally occurring radioactive materials such as radium have been used as radiographic sources from the earliest days of their discovery at the end of the nineteenth century. Since that time many industrial uses have been found for these x-ray emitters, as, for example, the detection of blow-holes and other flaws in metal castings.

In the field of medicine, however, the high penetrating power of these natural x-ray sources is far too great for radiography of the human body, both soft tissue and bone being traversed with almost equal facility. As a result of this no useful degree of contrast is registered on the photographic film. This situation, however, has been changed by the rapid post-war development of atomic physics, which has, amongst its many other achievements, made available a large number of artificial sources of x-radiation. Of these radioactive isotopes a few have penetrating powers low enough to produce satisfactory contrast as between bone and tissue.

Using one of these isotopes it is now possible to produce an extremely simple x-ray apparatus of such small weight and size that it can be carried around, together with a supply of films and cassettes, in a container little larger than a normal brief case. The apparatus is completely self-contained and requires no external power either from electricity mains or batteries. The possibilities of such an apparatus are many and varied. In field work, both military and civil, uses will at once come to mind, especially the examination of casualties. In the veterinary world also this type of apparatus may be of considerable value due to the ease with which it can be handled. Other uses already found include the radiography of old paintings and of porcelain figures.

To extend the usefulness of such an apparatus a portable film-developing outfit would be of great value and already a process has been developed by Picker-Polaroid, which has considerable possibilities in this field.

CHOICE OF SUITABLE ISOTOPES

We have seen that the primary requirement of a radioactive isotope for medical use is that its energy should be low enough to give good contrast; energies in the region of 50 kV have been found to be suitable. A secondary requirement is that the isotope should have a reasonably long half-life in order to avoid frequent replacement and recalculations of dose-rate as its strength decays. Two isotopes in particular have been investigated which

Fig. 3.—Radiograph of mandible of dried skull taken with a fragment of thulium-170 in mouth.

(a)

(b)
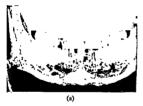
Fig. 4 —Mandible. Comparison of (a) x-ray and (b) thulium-170 radiographs.

demountable and packs into a comparatively small space for transportation.

RADIOGRAPHIC EXPERIMENTS

The dose-rate from sources at present available does not approach that produced by conventional x-ray apparatus. As a result, we must use shorter focal distances and longer exposure times than in normal practice. To reduce the time of exposure to the minimum, fast films and intensifying screens are necessary and for this purpose we have used Ilford double-coated 'red seal' films and Ilford H.V. screens. For the type of work on which these sources are likely to be used the results are very satisfactory despite the loss of definition inherent in this combination of films and screens. A stationary Lysholm grid was tested with various objects but no improved definition could be demonstrated and the exposure times were doubled. A moving Bucky diaphragm resulted in a slight improvement but again at the expense of increased times of exposure.

We have seen that thulium-170 sources have a bremsstrahlung component with a mean energy of about 150 kV. This comparatively hard radiation results in a noticeable loss of contrast; in this respect thulium-170 compares unfavourably with xenon-133 which has no significant radiation above 85kV. As the amount of bremsstrahlung present is directly related to the volume of thulium in the source, we should expect a small fragment of thulium-170 to give very much better con-

Ireland, 1956); as little as 3 mm. of this material is sufficient for shielding purposes when the source strength does not greatly exceed the above figures.

It will be obvious that there are many simple methods of making a suitable shutter, the precise approach depending on the application in mind. One solution, designed for clinical use, is illustrated in fig. 1. It was originally intended to contain the double-sealed thulium-170 disc but it can easily be adapted to hold xenon-133 sources.

The dual capsule holding the thulium is connected to a Bowden cable leading to the operating plunger, the cable length being about 25 cm The far end of the capsule is covered by a gold disc, 3 mm. thick, which in the closed position lies within the end of a gold cylinder of 3 mm wall thickness. This gold cylinder is terminated
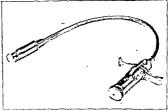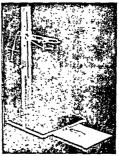
FIG. 1.—Gold-shielded clinical source holder.

by a perspex dome into which the capsule and its gold disc move when the plunger is operated. This is the exposed position and it will be seen that a 'lighthouse' beam or, more precisely, disc of radiation is emitted.

A possible application for this type of source holder is internal radiography, the source being inserted into various body cavities with the film outside. In this way unusual views can be taken, one advantage being that the normal superimposition of structures is avoided. Difficulties arise, however, in that the source, due to its close proximity to body tissue, causes a very high surface dose-rate: for example, the dose-rate due to a particular xenon-133 source was 250 r/minute at 0.5 cm. Another problem lies in the fact that because of the short focal distances involved there will be considerable geometrical distortion.

For normal use this source holder can be mounted on a stand such as that shown in fig. 2. This allows for variation of source-film distance and by sliding the source along the horizontal arm it can be centred over cassettes of various sizes The illustration shows a 12 inch (30.5 cm.) × 10 inch (25 cm.) cassette in position. The whole stand is

FIG. 2.—Gold-shielded clinical source holder on demountable stand, and cassette holder.

cannot be produced with sufficient strength to give short exposure times; the radiograph of fig. 3 required an exposure of about 10 hours.

The presence of bremsstrahlung, together with the fact that thulium-170 is not such a safe material to handle, has led to the conclusion that xenon-133 is a more satisfactory source. Its disadvantage of a short half-life of 5.27 days can be set against the fact that due to the very large amounts available in atomic reactors it should be a simple matter to arrange for a supply of replacement sources at low cost. Some examples of radiographs made with xenon-133 are shown in fig. 5 and 6. The source used had an initial dose-rate of 70 mr/minute at 30 cm., this being the source-film distance at which most of these pictures were taken. It will be seen that radiography of the extremities is possible with exposures as short as 3 seconds when sources of this magnitude are employed. The radiograph in fig. 7 is included as one example of the many non-medical uses for these radioisotope sources.

FIG. 7.—Radiograph of porcelain ornament taken with a fragment of thulium-170.

A useful application would be in dental radiography, the method being to place the radioactive source in the centre of the mouth with the film wrapped round the outside of the face. Advantages would be orthogonal views of the full complement of teeth at one exposure instead of the many separate exposures required when using normal techniques. The source-film distances are of course very small, and this results in geometrical distortion from two causes; one due to the thickness of the teeth and the other to the finite size of the source. With sources of 2 mm. diameter the results have so far not been satisfactory but it is hoped that with a reduction to 0.5 mm. or less some useful results may be obtained. In this case the unavoidable reduction of source strength with size is offset by the short source-film distances.

A comparison of fig. 4 (a) and 4 (b), both of which were made with the same geometrical conditions and films, will make clear the difference in quality between thulium-170 and normal x-ray tube radiations. This difference is probably due to the continuous nature of the tube spectrum as compared with the mainly monochromatic line structure of the thulium-170. Here we may note that an x-ray tube at, say, 75 kV has an effective

trast than larger sources. That this is so can be seen by comparing the radiograph in fig. 3, taken with a small fragment, with that in fig. 4 (b), taken with a 2-mm. disc source. It is unfortunate that fragmentary sources

(a)   (b)
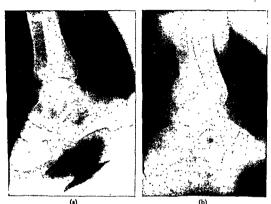
FIG. 5.—Xenon radiographs: (a) normal ankle, (b) fractured ankle.

(a)   (b)
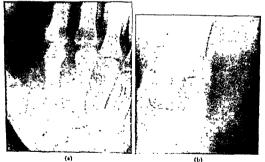
FIG. 6.—Xenon radiographs: (a) hand, exposure 3 seconds, (b) elbow, exposure 5 seconds.

HIGH CONTRAST: A POTENTIAL SOURCE OF RADIOGRAPHIC ERROR

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IN most discussions of radiographic principles great stress is laid on the triad of detail, definition and contrast, and the student is often left with the concept of the interlinking of the three, and a feeling that, as increasing contrast tends to improve definition and hence the visualisation of detail, contrast is desirable *per se*—in other words, the higher the contrast or density range the better the radiograph. As he gains experience he realises that this is not necessarily so and that in many cases, especially in techniques such as angiography, or in fine marginal bone detail, the more evenly graded film with a long tone scale will be far more informative. The radiologist who uses a local bright light as a means of viewing dense portions of a radiograph is effectively increasing the tone scale, though often he does not realise it, and it is only when the question of reproduction of the film with a high density range arises that the disadvantages become really apparent.

THE INCIDENT IMAGE ("RADIATION RELIEF")

In this paper distinction is made between the distribution of intensities incident on the film, which is controlled by the absorption differences within the subject, and the photographic record of that distribution—the radiograph. The incident intensity distribution may be thought of as recorded by an infinite number of minute ionization chambers, which we may imagine to be placed in the plane of the film or, in the case of fluoroscopy, of the screen.

If these incident intensities be represented as rods, of height proportional to the intensities, erected over the plane of the film, a kind of mountain relief map would be formed. The ratio of two neighbouring intensities (that is, two neighbouring heights) would represent the *radiation contrast* at that particular boundary. In map-making terms, the contour interval and the horizontal equivalent of the slopes would be employed as a measure of definition at the boundaries involved. The incident image of a stationary subject formed by a point focus would be free from any penumbra and in this sense would be infinitely sharp, and in the same way a sharp edge,

such as the boundary between a steel ruler and more translucent material, would be seen on the map as a vertical "cliff". In the case of the human body such complete discontinuities of intensity do not occur, *e.g.* at the boundary between bone and tissue where the bone thickness along the path of the radiation is varying.

Now, in this connection it is most important to realise that a wedge of any material will produce the same unsharp image (or slope on the map) as a blur and the two "unsharpnesses" cannot be distinguished on the image. Anatomical blur and technical blur (such as focal blur) which produce the same intensity distribution may be termed "equivalent blurring".

FIG. 1.

Intensity distribution across a slit 0 35 mm wide of the incident blue light emitted by two types of intensifying screen.

Since the photographic blur is the recorded image of the incident blur it seems appropriate to base further discussion on one specific type; we have chosen that caused by the use of an intensifying screen.

Fig. 1 shows schematically the incident intensity distribution of the fluorescent light emitted by a

average radiation energy considerably above this, in the region of 100 to 120 kV. Nevertheless, in spite of this poorer quality, radioactive isotopes, whilst not suitable for fine detail examination, have shown that for gross changes, e.g. dislocations and fractures, they may have a useful role to play in human radiography.

HAZARDS

We have seen that the radiation from these isotopes is easily screened and reduced to a safe level. There is, however, a serious risk from accidental ingestion of the active material. Thulium-170 is generally produced from sintered thulium oxide so that the possibility of some of the powder escaping from its capsules must be guarded against. Thulium-170 is a bone-seeker with an effective biological half-life of 60 days, the maximum safe body burden is only 4 microcuries (recommendations of the International Commission on Radiological Protection, 1955). This amounts to less than 0.1 micrograms of the material, underlining the need for perfect sealing. From this point of view it would be better to use the metal instead of its oxide (Untermyer *et al.*, 1954).

Xenon-133 is less dangerous because of its short half-life, the maximum permissible air concentration is 4×10^{-6} microcuries/ml. and the maximum body burden is 320 microcuries (Tobias *et al.*, 1949). A further safeguard is the fact that, in common with the other rare gases, it is chemically inert and not easily absorbed in the body.

References

Bergstrom, I. (1951): 'Manne Siegbahn Commemoration Volume', Uppsala, p. 395.

[illegible] 109.

[illegible]   on Radiological Protection

[illegible]   sources using xenon 133,
                                                      Research Establishment
(unpublished).

Tobias, C. A., Jones, H. B., Lawrence, J. H., and Hamilton, J. G. (1949): *J. clin. Invest.*, 28, 1375.

Untermyer, S., *et al.* (1954): *Nucleonics*, 12, 35.

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FIG. 1.

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Since the photographic blur is the recorded image of the incident blur it seems appropriate to base further discussion on one specific type; we have chosen that caused by the use of an intensifying screen.

Fig. 1 shows schematically the incident intensity distribution of the fluorescent light emitted by an

intensifying screen exposed under a slit 0·3 mm wide cut in an opaque metal plate. The two curves are those for normal (all-purpose) and high-definition screens, the former producing a curve with flatter slopes and more depression of intensity within the slit. It will be seen that light is scattered outside the highlight of the slit area into the surrounding shadow region, and that radiation is also reduced within the area of the slit, the reduction increasing with increasing unsharpness. The pecked square-topped line shows the ideal distribution obtained when no screens are used.

PHOTOGRAPHIC BLUR

Extension of the image with increasing radiographic exposure

From Fig. 1 it is obvious that short radiographic exposure* of the incident blurring will record only the region of high intensities within the slit area, for the low intensities will be below the photographic threshold of the film. Increasing exposure will
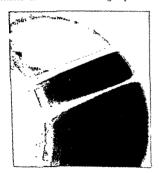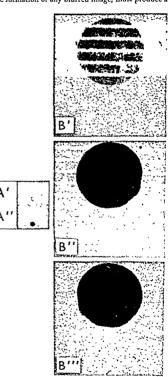
FIG. 2.

The shadow of a driving belt, of which the lower section was vibrating, shown against the blackenings produced by a sector-wheel

therefore record regions whose light is scattered farther away from the edge. Thus the width of a blurred image increases with exposure. With a sharp image, however, the width of the photographic image is invariant with exposure. (Irradiation and

* The term "exposure" is used in this paper to mean the dose in milliröntgens to which the particular region is exposed.

halation effects within the photographic emulsion, which are small, are neglected.)

The spread of light from the highlights into the neighbouring shadows, which is inseparable from the formation of any blurred image, must produce a

FIG. 3.

Facsimiles of two radiographs of a stationary grid, made through a brass bar through which a 2 mm hole has been drilled. A″ was given three times the exposure of A′. B′ and B″ are enlargements ×15 of A′ and A″. B‴ is a similar enlargement ×15 of a radiograph made during movement of the grid through 0·2 mm.

corresponding narrowing of the adjacent shadow areas. Fig. 2 is an example of this constriction, which is seen to increase with exposure: a non-screen film was placed behind a rotating lead sector-wheel, such as is used in sensitometric work, and exposed to X rays to obtain the characteristic curve of the film. One side of the transmission belt between the motor and the wheel happened to vibrate; the unsharpness of the shadow of the vibrating belt shows against a range of photographic densities. The blur is most obvious where the shadow shows with low contrast against low densities, whereas the boundary appears sharper though more constricted in the region of high density and high contrast or density range.

Fig. 3 shows the effect on resolution of high photographic densities near small shadow areas. A Schönander-Lysholm grid (27 lines/cm; line width 0·13 mm) has been used to produce a fine incident pattern, Figs. 3A' and 3A" show facsimiles of two

FIG. 4.

Radiograph of a metal clamp holding a sheet of aluminium 1 mm thick. The clamp was moved 4 mm during the exposure.

FIG. 5.

Comparison of soft and hard-ray techniques. In the upper graphs are shown the dose gradients; the lower unbroken curves show the corresponding photographic response. The pecked curve "eye" indicates the visual effect of the density gradient

FIG. 6.

The effects seen in Fig. 5 applied to two adjacent edges, showing the construction of the image in the soft ray technique.

FIG. 7.

High and low contrast radiographs of an aluminium step-wedge.

radiographs of the stationary grid, made on a non-screen film: the radiographic exposure in 3A' was one third of that in 3A'', and the radiation contrast, by appropriate choice of radiation, was four times that in 3A''. Figs. 3B' and 3B'' show enlargements ×15 of these radiographs. Fig. 3B''' is a similar enlargement of a radiograph of the grid which has been moved 0·2 mm during the exposure. The Lysholm grid is recorded partly through a circular hole in a brass bar and partly through the bar itself. Satisfactory resolution of the stationary pattern is observed within the holes, the density of the original radiograph in the hole of 3A'' being 1·4. Resolution of the grid pattern is not obtained under the metal bar in 3A', since here so much radiation is absorbed by the metal that the pattern is imaged within the toe of the characteristic curve, where contrast is too low and thus resolution is inadequate. The enlargement of the radiograph of the moving grid (3B''') shows how heavy exposure can obliterate a fine pattern when the incident image is blurred. Because bevelled edges will produce the same incident radiation pattern as blur due to physical factors, Fig. 3B''' reminds us of the small range of resolution available for a fine pattern whose boundaries are intrinsically unsharp.

The high-contrast image

Fig. 4 illustrates the gross impairment of resolution of large wedge-shaped shadows by excessively high contrast, and the production of "spurious sharpness". A metal clamp holding a sheet of aluminium 1 mm thick was moved 4 mm during the exposure, in a direction at right angles to its length. Where the radiation contrast is high, *i.e.* where the clamp shows against air, the blur or "movement wedge" is almost invisible; where the shadow of the clamp shows against the aluminium sheet, and thus where the radiation contrast is lowered, the "wedge" (an important detail) is very obvious, for it is 4 mm wide. If a similar clamp were constructed with a 4 mm bevel the wedge would lead to an identical

FIG. 8.

Low contrast radiographs of the step-wedge beneath (A) paper, (B) the "lung structure" of sponge, (C) the "lung" and a stationary grid.

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intensity distribution without any movement, but in the high contrast region the equivalent blur would be lost; we should produce a sharp radiograph of an unsharp object! The two halves of Fig. 4 may be considered as two separate radiographs, the high contrast section on the left being made with a lower kilovoltage than the section on the right.

The essential practical importance of the preceding point is that the intensity distribution from the centre toward the circumference of the image of a tube, such as a vessel or ureter, varies in much the same way as in the case of wedges of circular or biprismatic section. All that has been said of the faithful rendering of gradual transition and of spurious sharpness therefore applies in clinical practice, and it further applies when the question of reproduction arises, for excessive contrast and falsification of the density range can only too easily be introduced at this stage also. This will be discussed later.

Closer analysis of the unsharp image

In order to increase our understanding of what occurs in the high and low contrast images of unsharp boundaries, Figs. 5 and 6 may help us to visualise the situation. Suppose two images of an unsharp boundary 0·4 mm in extent be produced by focal unsharpness, using high kilovoltage in one case and low kilovoltage in the other. Let the tube tension be so adjusted that the low kilovoltage produces three times the radiation contrast of the high kilovoltage, while the shadows are identically exposed on both radiographs. Then the highlights, which are left to take care of themselves, will receive a much higher dose with soft radiation than with hard, and in this way a steep incident dose gradient will be set up by the soft rays, while that from the hard rays will be much flatter. In Figs. 5 and 6 the shadow dose is assumed to be 20 mr, and the neighbouring highlights receive, say, 120 mr with soft rays but only 40 mr with hard (the upper graphs in Fig. 5).

The photographic image of these incident gradients is formed within the characteristic curve of the emulsion employed. The lower graphs in Fig. 5 show photographic density plotted against dose; for hard rays, where the dose range is small, the image falls within the "straight-line" part of the characteristic curve and is recorded proportionately, as a line very nearly straight; using the longer dose range of the soft ray technique, however, the record is no longer proportional.

The response of the eye also enters into the picture at this stage. Over the smaller density range of the hard ray image the eye roughly follows the

photographic density curve, but over the longer range of the soft ray image it fails to respond to the higher densities, as shown in the pecked line labelled "eye". The edge is thus seen rather sharper but displaced toward the shadow (see also Figs. 2 and 4). *Steep density gradients thus simulate good definition.* Almost all handbooks of radiography say, if only by implication, that this "improvement" of definition by increased contrast and density range is a good thing; we consider that it may in fact be frankly spurious.

Turning to Fig. 6 it is seen that if two highlights lie sufficiently close to each other, leaving a small shadow area between them, the superimposition of the two "eye" curves is apt to produce a closing up of the small area. The resultant of the two "eye" curves is shown as a dotted line, and it is seen that the high contrast image of a small shadow detail is more liable to closing up than · when moderate contrast is employed.

ON OPTIMUM CONTRAST
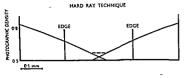
While we have given a warning that excessive contrast may have a deleterious effect on the rendering of detail, it must also be realised that too low a contrast may fail to bring out small absorption differences. Given an ideally sharp image boundary, the minimum perceptible photographic density difference is of the order of 0·02. In medical radiography a much greater difference will be required, particularly where the contrast of interest is superimposed, on a pattern which may distract the observer's interest from the significant detail.
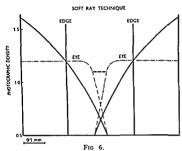
FIG. 9.
The "lung" and stationary grid. Compare this with Fig. 8.

Figs. 7, 8 and 9 are facsimiles of radiographs of an aluminium wedge with 1 mm steps, ranging from 1 to 10 mm. Figs. 7A and 7B are high and low contrast radiographs obtained by varying the quality of radiation; the radiation contrast per step is about 25 per cent in 7A and 6 per cent in 7B. It is

HARD RAY TECHNIQUE

SOFT RAY TECHNIQUE

Fig 6.

The effects seen in Fig. 5 applied to two adjacent edges, showing the construction of the image in the soft ray technique.

Fig 7.

High and low contrast radiographs of an aluminium step-wedge

radiographs of the stationary grid, made on a non-screen film: the radiographic exposure in 3A' was one third of that in 3A'', and the radiation contrast, by appropriate choice of radiation, was four times that in 3A''. Figs. 3B' and 3B'' show enlargements ×15 of these radiographs. Fig. 3B''' is a similar enlargement of a radiograph of the grid which has been moved 0·2 mm during the exposure. The Lysholm grid is recorded partly through a circular hole in a brass bar and partly through the bar itself. Satisfactory resolution of the stationary pattern is observed within the holes, the density of the original radiograph in the hole of 3A' being 1·4. Resolution of the grid pattern is not obtained under the metal bar in 3A', since here so much radiation is absorbed by the metal that the pattern is imaged within the toe of the characteristic curve, where contrast is too low and thus resolution is inadequate. The enlargement of the radiograph of the moving grid (3B''') shows how heavy exposure can obliterate a fine pattern when the incident image is blurred. Because bevelled edges will produce the same incident radiation pattern as blur due to physical factors, Fig. 3B''' reminds us of the small range of resolution available for a fine pattern whose boundaries are intrinsically unsharp.

The high-contrast image

Fig. 4 illustrates the gross impairment of resolution of large wedge-shaped shadows by excessively high contrast, and the production of "spurious sharpness". A metal clamp holding a sheet of aluminium 1 mm thick was moved 4 mm during the exposure, in a direction at right angles to its length. Where the radiation contrast is high, *i.e.* where the clamp shows against air, the blur or "movement wedge" is almost invisible; where the shadow of the clamp shows against the aluminium sheet, and thus where the radiation contrast is lowered, the "wedge" (an important detail, for it is 4 mm wide) is very obvious. If a similar clamp were constructed with a 4 mm bevel the wedge would lead to an identical

Fig. 8.

Low contrast radiographs of the step-wedge beneath (A) paper, (B) the "lung structure" of sponge, (C) the "lung" and a stationary grid.

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In Fig. 8 the effect of superimposition of other media is seen. In Fig. 8A the wedge was placed under layers of paper which produced roughly the same overall absorption of radiation as did 4 cm of sponge in Fig. 8B, employed to simulate lung structure and radiographed at low contrast. Here only the three thinnest steps are visible, their image being accommodated on the straight-line part of the characteristic of the film. Fig. 8C again shows the "sponge-lung" as in Fig. 8B, but here a Schönander-Lysholm grid has been interposed between the sponge and the film. Fig. 9, page 485, is identical with Fig. 8C. It is fascinating to speculate how the uninitiated radiologist would report on it were it isolated from the stages shown in Fig. 8. It would surely surprise him to know that Fig. 9 shows the same subject as Fig. 8.

Experiments such as these, together with careful practice, suggest that a photographic "contrast" (density range) between 0·5 and 1·2 might be
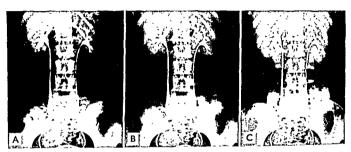
Fig. 12.
A retrograde pyelogram: (A) on hard paper; (B) on soft paper; (C) a masked print on hard paper, wherein the apparent narrowings seen in (A) are avoided.
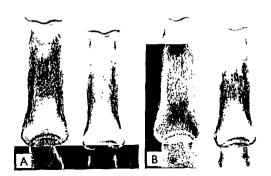
Fig. 13.
Apparent sharpening of internal structure and gross loss of marginal detail by the soft ray technique.
Hypertrophic pulmonary osteoarthropathy.

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obvious that in 7B the exposure under the thick part of the wedge is insufficient to reveal the absorption differences per step, for here they are recorded on the toe of the characteristic, where the gradient is small.

Fig. 10

Fig. 11.
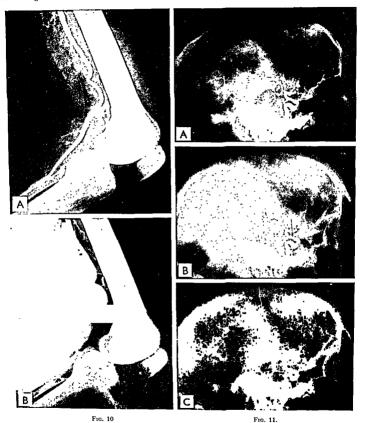
Fig 10 Accentuation of an apparent gap in the artery by the use of excessive contrast. Note the loss of collaterals in (B).

Fig. 11 A carotid arteriogram printed on (A) hard paper, with gross loss of detail; (B) on soft paper, which still does not retain structure in the petrous area and in the face; (C) a masked print where maximum detail is held.

Accelerated Ageing – a Long Term Effect of Exposure to Ionizing Radiations

By PETER ALEXANDER

Introduction

One of the major difficulties in the search for the physiology, or if senescence is considered as a disease, the physio-pathology of ageing is that the process cannot be induced by controlled methods in the laboratory. So long as studies have to be confined to watching the symptoms and recording the changes which occur, it will remain difficult to distinguish primary from secondary effects. This is particularly serious on the biochemical level and even now there is no evidence whether there is a biochemical lesion associated with senescence. As it is, more information has probably been gained from the experiment of C. M. *McCay* who was able to prolong the total life span of rats by maintaining them on a severely restricted diet, than from any other experimental investigation on ageing. A deliberate interference with the spontaneous process had been made and as a result some definite conclusions became possible. Unfortunately no process other than starvation has been found which will extend life and most of the information which can be obtained from this kind of experiment is probably available. In this paper I wish to propose a complementary approach, that of reducing the normal life span.

optimal. In the most desirable radiograph the overall tone range would be compressed, yet small absorption differences would be rendered as high photographic density differences. Various promising .... been made recently; ·· · is the best known. the use of television technique for diagnostic radiology in which the control of tone-volume would be possible. Spiegler and Juris (1931, 1933) have introduced a harmonising or masking process for the reproduction of radiographs, and developments of this are referred to by Ollerenshaw (1953). The uses of this procedure are, however, limited, for it increases the time, materials and labour required.

CLINICAL CONSIDERATIONS

In practice excessive contrast may lead to two undesirable results: the total loss of detail through "blacking out" of regions of low absorption, and the artificial sharpening already discussed. While in the radiograph itself some of the lost detail may still be observed by the application of a local intense light, in a reproduction it is inevitably lost. In Fig. 10 is shown the effect of (A) suitable radiation and (B) too soft radiation on a femoral arteriogram. In the example made with a high kilovoltage, the compression of the popliteal artery by soft tissues is clearly seen for what it is, while the excessive contrast in (B) produces the effect of a definite gap. At the same time many of the small collaterals have entirely disappeared. A similar effect, due wholly to reproduction procedure, is seen in Fig. 11, where the upper example (A) has been printed on paper of too high contrast, the centre example (B) on a soft grade (which still has not held the facial skeleton), and the lower print (C) is produced by masking and printed on a normal grade of paper. In this the whole range is preserved and the general balance is much more pleasing. The effect of using too hard a paper

is very similar to that produced by radiation of too low a kilovoltage.

Fig. 12 is an example of the production of unreal narrowings in tubular viscera, in this case the ureters, and the possibility of correction by the method of Spiegler and Juris (1931, 1933), using the masking frame described by Spiegler and Giles (1953). The prints are (A) on hard paper, (B) on soft paper, and (C) a masked print on hard paper. The same "closing up" as in (A) will be produced on the radiograph itself by the use of too low a kilovoltage or a film of too steep a gradation.

These effects are not confined to hollow viscera, and they appear in specialised processes. Radiographs were made, at differing kilovoltages, by the fine-grain method of Rowley and Fletcher (1951). Even with this essentially low contrast method of processing the fine marginal trabeculae of new bone in hypertrophic pulmonary osteoarthropathy (Fig. 13) may be entirely lost. The pair of radiographs were made at 90 kV (A) and 40 kV (B).

SUMMARY

Unsharp transitions in incident radiation relief may be due to anatomical and pathological conditions and should therefore be rendered visible in the final radiograph. The harmful effect of "sharpening up" the appearance of unsharp boundaries by excessive contrast is discussed, and the somewhat fallacious link commonly taught between detail, contrast and definition is shown to require qualification. Investigation of the lower limit of perceptible density difference under unfavourable conditions permit an estimate of optimum density range in practice. Some clinical examples are considered and the function of contrast in reproduction is pointed out.

REFERENCES

FLETCHER, D. E., and ROWLEY, K. A., *Brit J. Radiol.*, 1951, xxiv, 598.
MAYNEORD, W. V., *Brit. J. Radiol.*, 1952, xxv, 589.
OLLERENSHAW, R., *Brit. J. Radiol.*, 1953, xxvi, 73.
SPIEGLER, G., and GILES, P., *Brit. J. Radiol.*, 1953, xxvi, 130.
SPIEGLER, G., and JURIS, K., *Phot. Korr.*, 1931, lxvii, 4 *ibid.*, 1933, lxix, 36.

us right up against one of the focal questions of gerontology; what distinguishes a young from an old animal. The literature abonds with histo-pathological observations and it is quite possible for the experienced observer to give his impression from sections whether an animal seems to be old. But none of the changes are suitable as a test since they cannot be made quantitative. Even as purely subjective indications they are unreliable, since pathological changes predominate, which need not follow directly from the more fundamental biological and biochemical changes which initiate senescence. Apart from the sex organs physiological function has not been satisfactorily related with age; for example changes in capillary circulation and diffusion, though definite, cannot be used quantitatively. The position about biochemical tests, such as distribution of calcium and changes in connective tissues, is equally vague.

An approach which seems hopeful is based on the discovery by *F. Verzár* and co-workers, that with increasing age animals adjust themselves less readily to changes in environment.

Adaptation to reduced atmospheric pressure, where change in body temperature is used as a criterion (3), was found to be both slower and less complete with older rats. It is significant that the response to low oxygen tension of the bone marrow in increasing the haemoglobin level was not influenced by age, any more than was the rate of replacement and compensatory hypertrophy of the kidney and adrenals following partial removal by surgery (4). As in the case of reduced pressure, old animals were less able to maintain their body temperature when placed for some hours in an environment of low temperature (5). Both experiments suggest that ageing decreases the ability of the general metabolism to respond to a sudden demand. Psychological tests (6) which have shown that old rats re-learn less quickly than young ones can probably also be considered as lack of adaptation.

Perhaps the simplest criterion for ageing is the elastic behaviour of rat tail tendons; *Verzár* (7) found that the force with which the collagen contracts when heated above 62° C increased steadily with age. We have recently confirmed this observation fully and are investigating the chemical changes underlying it. Chemical changes which occur in collagen are unlikely to be a primary factor in bringing about the biological end effects commonly associated with ageing; consequently failure to observe a change in the physical chemistry in the collagen of an animal which has been irradiated need not

In a closely argued study based on statistical evidence *Hardin B. Jones* (1) develops the concept that life span depends on the general disease history. Each illness predisposes to the contraction of further disease and an illness of any kind has therefore an autocatalytic effect on the general state of health. The length of life is determined by this disease history which in its turn is largely governed by illnesses contracted early in life. In brief – a long life must be a healthy one, statistically speaking. No attempt appears to have been made to test this concept in the laboratory and in retrospect it seems strange that, with the exception of radiation, the influence of a succession of severe stresses on life span has never been examined*. For example, the experiment of determining how a severe infection checked with an antibiotic affects long term survival remains to be carried out.

A number of good experiments have however been done which show that the irradiation of the whole body with X- and γ-rays and neutrons decreases the life expectancy of animals which had not succombed to the more immediate effects of radiation sickness. The object of this paper is to show that some deductions can be made about normal ageing from existing studies in radiation biology; but, I hope above all to indicate that by learning about the factors which determine the ageing mechanism of ionizing radiations a new experimental approach to the study of the universal illness of senescence is available. Moreover the experiments are of a comparatively short term character, since an accelerated test is involved and the effect of different prophylactic measures and of a variety of post-irradiation treatments can be examined. A programme to determine the influence of different chemicals or other treatment upon spontaneous ageing would be very difficult with laboratory animals since each experiment would take at least two to three years.

Criteria for Ageing

How can one tell whether the process of premature ageing bears any resemblance to the spontaneous process? This question brings

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* *Casis* and *Healey* (2) report that injections of typhoid toxin and of nitrogen mustard at toxic levels failed to produce a marked shortening of life span, but their experimental data is not capable of revealing any but the most pronounced changes since many of the animals died immediately after the treatment and the survivors cannot be strictly compared with the controls.

melanocytes in general requires doses which would be lethal if given to the whole body. It is very striking how a white patch which appears some weeks after whole body irradiation disappears completely after some months.

In general an animal which has not succumbed in the first month will apparently recover completely and can after some weeks not be distinguished histologically from that of a nonirradiated control.

Yet this recovery is not complete and some hidden injuries remain, the most notable being an increase in the incidence of cancer and leukaemia months or years later. Two types of carcinogenic action must be distinguished; local irradiation with very high doses (many times greater than the LD 50 if given to the whole body) will produce relatively quickly tumours at the site of irradiation. Following whole body doses tumours appear towards the end of life and there are great variations within the same species from one strain to another, both with regard to the types of neoplasm and their frequency. In some strain whole body radiation produces leukaemia, in others ovarian tumours, while in others a wide spectrum reminiscent of spontaneous cancer in non-inbred strains is found. A notable feature is that genetic factors are very much more important in determining the cancer pattern after whole body than after high doses of local irradiation. For example, in one strain of mice a whole body dose of as little as 25 r (LD 50 is about 600 r) is definitely carcinogenic, while in others not even 500 r produced a detectable increase.

The "long term" effect of interest to gerontology is the shortening of life span. The first question is whether this is real or is merely a reflection of the carcinogenic action relatively late in life. There is one clear cut series of experiments (8), the relevant results of which are shown in fig. 1, which prove that even when allowance has been made for death from cancer the irradiated animals died sooner than the controls. It must be stressed that this applied to animals which were relatively susceptible to radiation carcinogenesis. The relative shortening of life span seems to be effected much less by genetic factors than the induction of cancer and in other strains the evidence for shortening of life span by death other than cancer is therefore even more convincing. Indeed many malignancies produced by irradiation are found so late in life that they do not seriously influence survival times.

imply that radiation does not simulate true ageing. But a positive
result would argue strongly for a close relationship. In the adapta-
tion experiments, on the other hand, a very fundamental change is
measured and one which is most unlikely to be a mere secondary
side-effect. In the present state of knowledge they are probably the
most critical quantitative measurements for ageing which can be
made to test whether a treatment such as irradiation which shortens
life span is related to normal ageing.

Shortening of Life Span by Irradiation

The effects produced by exposing the whole of an animal to
ionizing radiations fall broadly into three groups*; the immediate
effect, the long term effects and the genetic effects. The last only
become apparent in future generations and are not relevant to the
problem under discussion. Most of the immediate effects in small
mammals are noticed within a few days after irradiation. If the dose
is large enough, death from radiation sickness will occur on about
the 10th day in the case of mice and rats, although there are re-
markable variations between strains. With smaller doses recovery
from most of the symptoms of radiation sickness sets in quickly and
for most organs is complete in less than a month, as far as can be
judged histologically. Although many cells are killed they are
replaced so that the different organs regain their normal function.
When the dose is great (i. e. approaches the LD 50) then restitution is
incomplete, in a few special cases, the most important of which are
the reproductive organs. Temporary sterility is a general symptom
of radiation sickness, but with large doses there is sometimes no
recovery or more commonly there is permanent partial sterility,
revealed by small litter sizes. Greying and loss of hair by radiation
very strikingly demonstrates the power of complete recovery and
the fur returns to its original state even after wide spread epilation.
Greying of hair is also temporary and permanent destruction of the

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* In this discussion the effects produced by the so-called supra-lethal doses which kill
the animal within minutes or hours of the irradiation are not considered. The definition
of a lethal dose is necessarily arbitary since all exposures are believed to decrease life
expectancy. The magnitude of the lethal dose will depend therefore on the period of time
which is allowed to intervene between irradiation and scoring of death. In practice this
difficulty is not serious since radiation either kills a mammal within one to two weeks
from immediate effects or many months later from delayed effects. The usual definition
of the lethal dose as the LD 50 within 30 days is for most purposes unambiguous.

hundred roentgens. Clearly, the shortening of life span by radiation is not the result of a single severe stress – i.e. radiation sickness – and cannot be fitted into the theory of *Hardin B. Jones*, that life expectancy is determined by the number of serious illnesses throughout life. Although the existence of the effect is certain, quantitative data for life shortening by radiation is rather confused, since the influence of different regimes of irradiation have not been studied systematically. For example, neither the relation between dose and reduction in life expectancy nor the relative effectiveness of single and repeated irradiation have been established. All our information comes from data obtained incidentally in experiments designed for other purposes. *H. A. Blair* (9) has collected together the best of all the information available for mice and rats and has compared them by the expedient of expressing the dose not in roentgens but as the fraction of the LD 50 (30 days) which varied widely in the groups compared. Thus a dose equal to half that of the LD 50* (see fig. 3), shortened life span by 12%. With repeated small doses, much greater shortening in life span can be obtained (10) since the *total* dose received may be two or three times greater than the LD 50, if the successive irradiations are properly spaced. Most of the
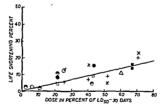
Fig. 3. Life shortening expressed as percentage of normal adult life-span of mice and rats following a single dose of X-r γ-rays. The dose is expressed as the percentage of the LD 50 (30 days) so that different strains can be compared. The different points represent different experiments (reproduced from *H. A. Blair* [9]).

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* Since the dose mortality curve for radiation is very steep less than 5% of the animals which have received this dose will have died acutely (i.e. within 30 days). This data is therefore not invalidated by selection until doses greater than 75% of the LD 50 are reached. The reduction in life span of the survivors from doses above LD 50 are very great, but the data may not be very meaningful since the groups will be atypical as a result of selection.
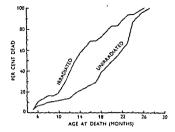
Fig. 1. Life-span of mice irradiated with 400 r of X-rays. In both the control and irradiated series all animals which had tumours at death were neglected (data from [8]).

The life span is shortened both by one single large dose, when the animal will have had serious symptoms of radiation sickness, as well as after repeated small doses which give rise to no immediate symptoms. Fig. 2 shows an early experiment where daily doses shortened life although the animals at no time showed signs of radiation sickness. Their bone marrows and intestines never suffered damage like that of animals which received a single dose of several
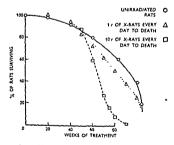
Fig. 2. Life-span of rats which had received small doses of radiation on every day of their lives (data from the Biological Effects of External Radiation [edit. by *H. A. Blair*], publ. *McGraw-Hill,* 1954).

of the tissues and fat deposition of irradiated animals assumes the appearance associated with old age much sooner than with non-irradiated controls. But the most impressive parallelism between ageing and long term radiation effects is in the pathological changes. Reference has already been made to the induction of cancer which can certainly be considered as a typical disease associated with ageing. In the normal population the incidence of cancer increases rapidly with age and radiation accelerates the time scale.

Much work has been done on the induction of cataract by irradiation. Although this condition is not a sequelae of whole body radiation like cancer, it shows that local effects of radiation which themselve do not shorten life produce cellular changes similar to those occurring spontaneously in old age. Loss of hair pigment and epilation and skin changes such as telangiectasia are also typical symptoms of old age which follow irradiation, often many years after a local irradiation given as therapy.

There are a number of reports, both from radiotherapists and animal experimenters, of changes in the kidneys (12, 13, 14) and the central nervous system (15, 16, 17) following a long time after irradiation, which seem to have special importance in relation to ageing since there are indications that (12, 16) the pathological conditions observed are secondary to sclerotic changes in the blood vessels.

Finally there is evidence that in addition to the immediate effect of relatively large doses there is progressive impairment of reproductive capacity following on protracted irradiation. For most organs damage is greatest when the radiation is given as a single dose but this effect is much less marked for the testis indicating that recovery processes are less effective (18), and long term effects become possible. For example, (10) a dose of 1 r of X-ray per day produced complete atrophy of the germinal epithelium of dogs at the end of 622 days, but did not damage significantly other radiation sensitive tissue like the bone marrow.

In the female the ovaries are very radiation sensitive – possibly more so than the male organs to a single dose – but they are much less sensitive to protracted doses (20, 21) which suggests that delayed effects will be less important. In relation to ageing it may however be relevant women who have received several hundred roentgen underwent an artifical menopause similar in most respects to that which normally occurs (22).

damage from each irradiation will be repaired before the next dose is received so that the level of damage is below that giving rise to radiation sickness in a severe form. When considered on the basis of dose only then repeated small doses are less effective in shortening life than one single dose although the exact factor cannot be evaluated, since in the experiments with divided doses there are so many variables that a relationship obtained from results of different experiments is meaningless.

Deductions have been made from this rather unsatisfactory data which exceed reasonable limits of scientific caution. For example, the statement that there is no threshold dose for life shortening and that every exposure is harmful in this connection cannot be accepted when it is realised that it is based on a compilation of different experiments such as that shown in fig. 3. Even more suspect is the statement that 1 r reduces the life of man by 10 days. This statement is based on the difference in life span of American doctors who had and who had not come into regular contact with X-ray machines. The former lived on an average some 5 years less and were guessed to have received the equivalent of some 150 r of whole body radiation. Hence 1 r loss of 10 days. Much more experimental data is necessary before extrapolation of this type become permissible.

Is the Radiation Effect Similar to Spontaneous Ageing?

This question cannot be answered other than by guesswork and intuition. Unfortunately the adaptation tests of ageing developed by *Verzár* (see p. 178) are too recent to have yet been applied to irradiated animals and no one appears to have made any attempt to compare natural and radiation aged animals by any tests which can be expressed quantitatively. We are therefore left with subjective impressions which are not very valuable in such a complex field. Right from the earliest publications (e.g. 10) pathologists have remarked on the fact that irradiated animals seem to be prematurely aged. Their coat goes grey and assumes the feel and general appearance of an old animal much earlier than that of a nonirradiated control*. According to experienced pathologists (e.g. 8, 10), the morphology

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* This greying is not related to the temporary or even permanent greying which follows shortly after a severe irradiation. This is an effect of radiation sickness which has disappeared long before the animals "age".

to measure the magnitude of this effect (25 and 26) its existence is revealed by an analysis of radiation data which was not intended to bear on this point (e.g. 27, 28). The best way of measuring residual damage is to give one or more large sublethal dose to a group of animals and determine the LD 50 at different times afterwards. The experiment must be designed so that the original population is not distorted by killing an appreciable number of animals by the conditioning dose.

The first experiment (26) using rats having an LD 50 for a single dose of X-rays of 600 r was as follows:

Animals were given 300 r, a dose which killed none of the rats, and were then irradiated with different doses to determine the LD 50 one, two and three weeks after the "priming" dose. The LD 50 was reduced to 430 r, 500 r and 530 r respectively. *Brauer* et al. (25) established that recovery of mice was complete after four weeks; three conditioning doses of 300 r were given at three weekly intervals without any fatalities; the LD 50 was then determined 4 weeks and 8 weeks after the last irradiation. It fell 644 r $\pm$ 15 to 544 r and 564 r $\pm$ 15 respectively.

Eividence that this reduction in LD 50 represents a true residual injury and not slow recovery is abundantly provided by data on the rate of recovery (e.g. 27, 28) using the "titration" technique described. By determining the LD 50 dose of X-rays at different times after the conditioning dose it was found that in the case of mice half of the recovery which could be obtained was achieved after seven days. Fig. 4 illustrates the situation in diagrammatic form.

Blair (29) and *Smith* (30) have extended the idea of residual damage to death from repeated and even continual irradiation. They postulate that an animal dies acutely (i.e. within 30 days) when it has sustained an amount of damage equal to that produced by a dose of X-rays which would kill a normal, i.e. not previously irradiated animal. This damage is made up of all the residual damage left from previous irradiations as well as the whole of the last dose received. Most of the available data on protracted irradiation can be satisfactorally accounted for on this theory. By assuming that the fraction is 20% and that restoration occurs exponentially and is half complete after seven days.

The simple treatment can however only be considered as a first approximation and there are indications that the proportion of

Although all these observations fall far short of proof that shortening of life span by radiation can be considered as an acceleration of the natural process, this concept provides a useful basis for further experiments.

Residual Damage

Experiments designed either to find out the mechanism by which radiation shortens life or how to reverse this effect may be simplified and very much speeded up if the lesions responsible can be identified with the residual or irreparable damage which is a characteristic feature of radiation injury. Following a dose of radiation to the whole body damage can be seen in mammals at many sites. Perhaps the earliest effect (about one hour after radiation) is a change in oxydative phosphorylation (23) in radiosensitive organs like the spleen. This is soon followed by changes in the blood pattern and all the other symptoms of radiation sickness. Recovery from some of these is extremely rapid; for example, damage to the gastrointestinal mucosa in mice and rats is very extensive as there is a complete cessation in mitosis less than one day after irradiation. But, regeneration sets in about three days afterwards and the organs appear quite normal about a week or so later, even in animals which have received a lethal dose and die soon after (i.e. within ten of irradiation). Permanent damage at these sites requires dose well beyond the lethal range (24). The position with regard to the radiosensitive organs such as the bone marrow and lymph glands is very similar. Recovery is apparently complete following doses up to the LD 50 (within 30 days) and the blood picture eventually returns to normal; only after repeated doses is there a permanent lowering in white cell count. Regeneration seems to be delayed longest in the reproductive organs and in hair (see page 179).

The existence of delayed effects such as cancer (see page 180) shows that recovery from a dose of radiation sufficient to produce severe radiation sickness is by no means as complete as the absence of lesions suggests. The incompleteness of this recovery can be revealed even without waiting for the appearance of the delayed effects since the resistance of an animal to a further dose of radiation is permanently reduced by earlier exposures. The magnitude of the residual or irreparable damage can be assessed by determining the reduction in the LD 50 dose, following earlier irradiations. Although only two experiments have been published which set out specifically

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ageing can therefore be obtained rapidly by measuring the LD 50 to X-rays.

In the previous section reasons for the view that radiation-produced ageing simulated the natural process were collected together. Very strong support for this view would be derived if the LD 50 for X-rays decreased with increasing age for ordinary (i.e. nonirradiated) animals. Natural ageing could then be equated with irreparable radiation injury. Surprisingly there were almost no data bearing on this point until a paper was published in 1956 (32). General agreement exists that young mice and rats up to eight or nine weeks old are more radiation sensitive than mature animals, but only isolated experiments were recorded on old animals. *Hursh* and *Casarett* (33) compared the LD 50 of six and sixteen month old rats and found that the LD 50 (X-rays) fell from 715 r to 600 r. These authors collected different data for rats in the literature and were able to construct a curve relating LD 50 with age which passed a distinct maximum at 100 days. The hypothesis that X-ray injury is equivalent to ageing injury seemed therefore to be strongly supported. However, a very recent experiment (33) of which the full details have not yet been published in full, shows that the LD 50 X-ray dose for mice does not fall significantly between 4 to 30 months (young mice are less resistant). It cannot be denied that the thesis of this paper that radiation effects can assist in the study of senescence would be greatly undermined if the LD 50 were not less for old animals. But, before abandoning the attractive view that death from old age, like death from repeated and protracted irradiation, occurs when the sum total of irreparable injuries sustained throughout life passes a given value, the possibility of modifying the theory must be considered. The test may not be straight forward since it may be necessary to wait for the onset of senescence before a reduction in radio resistance occurs. In other words a decrease in the LD 50 may only become apparent at an age at which the normal survival curve begins its steepening downward trend. This situation would arise if the accumulation of irreparable injury during normal life is an autocatalytic effect. That is, as irreparable injuries accumulate, so the ability to repair decreases and consequently stresses in later life are restituted to a smaller intent than when young. The same may also apply to the LD 50 for radiation, especially since there are indications that the irreparable fraction seems to increase with the size of the conditioning dose; see p. 187.

Fig. 4 Diagrammatic representation of the recovery of animals from a single dose of radiation on the assumption that recovery is (1) exponential with a half time of 7 days and (2) limited to 80% with 20% remaining permanently. The recovery is measured in terms of LD 50 If the initial LD 50 is R and the priming dose r, then LD 50 at different

$$\text{times} = R - \frac{\%\ \text{Recovery} \times r,}{100}$$

damage which is not repaired is greater following high doses than for low doses, but most of the experiments can be accommodated by a range from 10 to 20% for the irreparable fraction.

All the foregoing applies to sparsely ionizing radiations such as X- and γ-rays. More densely ionizing radiations, such as those produced by neutrons, are more effective, and the LD 50* is considerably lower. There are indications (31) that recovery from a sublethal dose is much less complete and the irreparable fraction may be as much as 50%. It would be most valuable to have more information about the long term injuries following irradiations with fast neutrons, since studies so far published are almost entirely confined to carcinogenesis and induction of cataract.

Residual Damage and Ageing: Although the evidence falls far short of proof all the data is consistent with the view that the permanent damage following irradiation revealed by shortening in life span (see page 182) is also responsible for reduction in the acute lethal dose. As far as can be judged both are proportional to radiation dose and of the same magnitude; that is, permanent injury corresponding to 20% of the LD 50 shortens life expectancy by approximately the same amount. A value for the extent of radiation-accelerated

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* The dose must be expressed in an energy unit such as the rad for comparison between different ionizing radiations 1 rad = 1 r (very nearly) for X-rays

ageing can therefore be obtained rapidly by measuring the LD 50 to X-rays.

In the previous section reasons for the view that radiation-produced ageing simulated the natural process were collected together. Very strong support for this view would be derived if the LD 50 for X-rays decreased with increasing age for ordinary (i.e. nonirradiated) animals. Natural ageing could then be equated with irreparable radiation injury. Surprisingly there were almost no data bearing on this point until a paper was published in 1956 (32). General agreement exists that young mice and rats up to eight or nine weeks old are more radiation sensitive than mature animals, but only isolated experiments were recorded on old animals. *Hursh* and *Casarett* (33) compared the LD 50 of six and sixteen month old rats and found that the LD 50 (X-rays) fell from 715 r to 600 r. These authors collected different data for rats in the literature and were able to construct a curve relating LD 50 with age which passed a distinct maximum at 100 days. The hypothesis that X-ray injury is equivalent to ageing injury seemed therefore to be strongly supported. However, a very recent experiment (33) of which the full details have not yet been published in full, shows that the LD 50 X-ray dose for mice does not fall significantly between 4 to 30 months (young mice are less resistant). It cannot be denied that the thesis of this paper that radiation effects can assist in the study of senescence would be greatly undermined if the LD 50 were not less for old animals. But, before abandoning the attractive view that death from old age, like death from repeated and protracted irradiation, occurs when the sum total of irreparable injuries sustained throughout life passes a given value, the possibility of modifying the theory must be considered. The test may not be straight forward since it may be necessary to wait for the onset of senescence before a reduction in radio resistance occurs. In other words a decrease in the LD 50 may only become apparent at an age at which the normal survival curve begins its steepening downward trend. This situation would arise if the accumulation of irreparable injury during normal life is an autocatalytic effect. That is, as irreparable injuries accumulate, so the ability to repair decreases and consequently stresses in later life are restituted to a smaller intent than when young. The same may also apply to the LD 50 for radiation, especially since there are indications that the irreparable fraction seems to increase with the size of the conditioning dose; see p. 187.

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Although the relation between irreparable damage following whole body irradiation with X-rays and senescence is tenuous, an investigation designed to determine the nature of this irreparable damage can be considered as a useful approach to the physiology of ageing changes, since there are so few other experimental possibilities of doing so.

There are several kinds of post-radiation changes which research in radiobiology suggests could give rise to irreparable damage. The incorporation of abnormal cells (or cell constituents), produced either during the initial injury or in the subsequent recovery phase, into organs which histologically appear to have returned to normal could be most harmful. The development of radiation cataracts may be traced to the deposition of fibres by cells which were killed by radiation. Imperfect removal of the debris interfered with normal replacement. Even the induction of tumours could arise in this way since *Oppenheimer* et al. (34) have shown that the subcutaneous implantation of any foreign substance, so long as it is in the form of a flexible and impermeable film, will produce tumours. The possibility of chemical carcinogensis has been excluded in these experiments and the effect must be due to some physical factor, such as perhaps hypoxia next to the film. It seems possible that radiation injuries might be followed by the deposition of some membrane-like structure which would then induce tumours in the same way as polymer films.

The preferential destruction of stem cells may produce an imbalance in rapidly dividing tissue which may not become apparent for a considerable time. Thus after radiation the exact time cycle which regulates the release of cells from bone-marrow may be permanently impaired. Granulocytes, which can be recognised histologically as immature, are present in the blood stream for some weeks after irradiation and this shows that at a time when the bone-marrow had become repopulated and looked apparently normal, it was not functioning quite normally. Some such defect may persist and it would not be recognised anatomically if the extent of maturation was only slightly effected. Yet the biochemical changes might be very great and reduce the resistance of the animal.

So far very little attention has been given to the damage done by radiation to capillaries and blood vessels. Increased fragility has

been noted but no detailed histological studies have been performed. The walls of these capillaries must be undergoing replacement just like the lining of the stomach and intestine. We know that radiation greatly injures these two organs by preventing the replacements of cells in the mucosae. An exactly similar situation probably exists with regard to the blood vessels and it is not inconceivable that these will undergo changes which may result in the same sort of injuries as the sclerotic changes associated with normal ageing.

Finally, while damage to the nucleus of cells may give rise to the genetic (somatic· mutations?) changes responsible for the appearance of tumours, damage to the cytoplasm may result in biochemical deficiencies which cannot be recognised histologically.

Localisation of Residual Damage: The first problem to decide is whether the reduction in acute lethal dose is due to a general failure to compensate for stresses of all kinds or whether specific organs are involved. It may be that the residual injury represents a "fatigue" of the recovery mechanism in which case the resistance of the animal to most types of poison would be reduced by an irradiation given at least six weeks earlier. This could be tested for by determining the difference before and after irradiation in the LD 50 for a number of poisons which are known to cause death in different ways.

Biochemical Lesion: If experiments of this type fail to indicate that the irreparable damage can be associated with any particular organ the cause for the reduced capacity to overcome severe stress must be the result of some general cellular injury. Since no visible damage persists attention should be focussed on some biochemical deficiency. As yet all the experiments on the pathological biochemistry of irradiated animals have been confined to the period of acute radiation sickness; no attempts appear to have been made, except for the induction of cataract, to correlate the long term effects with a biochemical change. In the absence of such data the number of possibilities is huge but the range can probably be narrowed by studying how external factors, which are known to influence the immediate consequence of radiation, effect the amount of residual injury produced. For example, the administration of cysteine or β-mercapto-ethylamine immediately before exposure doubles the radiation resistance of mammals as judged by the LD 50 (within 30 days). It is widely believed (see for examome 35) that this effect is brought about by the protection of the mechanism which accelerates the regeneration of haemopoietic tissues. In general the chemical

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There are several kinds of post-radiation changes which research in radiobiology suggests could give rise to irreparable damage. The incorporation of abnormal cells (or cell constituents), produced either during the initial injury or in the subsequent recovery phase, into organs which histologically appear to have returned to normal could be most harmful. The development of radiation cataracts may be traced to the deposition of fibres by cells which were killed by radiation. Imperfect removal of the debris interfered with normal replacement. Even the induction of tumours could arise in this way since *Oppenheimer* et al. (34) have shown that the subcutaneous implantation of any foreign substance, so long as it is in the form of a flexible and impermeable film, will produce tumours. The possibility of chemical carcinogensis has been excluded in these experiments and the effect must be due to some physical factor, such as perhaps hypoxia next to the film. It seems possible that radiation injuries might be followed by the deposition of some membrane-like structure which would then induce tumours in the same way as polymer films.

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So far very little attention has been given to the damage done by radiation to capillaries and blood vessels. Increased fragility has

the symptoms of senility to appear prematurely. Although the data
available is fragmentary and not very compelling it does suggest
that the artificially induced ageing bears a close similarity to the
spontaneous process. Studies with irradiated animals may assist in
obtaining an understanding of the fundamental biologically changes
of senescence. The very long period required for each experiment
makes it very difficult to study the influence of different factors on
normal life-span and the use of ionizing radiations may make it
possible to carry out experiments which will provide similar infor-
mation but more quickly. After an irradiation in which the whole
body has been exposed there is widespread cellular damage but
repair and restitution is rapid and in general no injuries can be seen
by the techniques so far employed some two months later in the
case of small mammals. Yet some hidden injury remains permanent-
ly and can be measured by a reduction in radio sensitivity. The re-
duction in life span of irradiated animals can probably be identified
with this residual damage.

Consequently by measuring residual damage, which can be
done relatively simply from the changes in LD 50, the influence of
different factors on radiation-induced ageing can be quantitatively
assessed. Experiments are suggested by which information about the
nature of the residual damage and its site might be obtained. In
view of the general similarities such knowledge may be of direct
value in determining the physiological and anatomical changes
which give rise to senescence. From the background of research into
radiobiology the possible ways by which irreparable damage could
arise are reviewed and compared with changes seen in normal ageing.
By using the large and rapidly increasing volume of research into
the long-term effects of ionizing radiation experimental gerontology
may benefit greatly.

Zusammenfassung

Eine Spätwirkung der ionisierenden Strahlung auf den Körper ist die Verminde-
rung der Lebensdauer. Die karzinogene Wirkung solcher Strahlung kann aber nur einen
kleinen Teil der Zunahme der Absterbegeschwindigkeit erklaren. Der Verlauf des
Todes bei einer bestrahlten Gruppe von Versuchstieren kann am besten als beschleu-
nigtes Altern charakterisiert werden. Die Strahlung hat ein zu frühes Auftreten der
Symptome der Senilität verursacht. Trotzdem die zur Verfügung stehenden Daten noch
fragmentarisch und nicht sehr zwingend sind, sprechen sie doch dafür, daß eine weit-
gehende Ähnlichkeit mit dem spontanen Alternsprozeß besteht. Untersuchungen mit
bestrahlten Tieren können deshalb benützt werden, um die fundamentalen biologischen
Veränderungen des Alterns zu verstehen. Während sonst die lange Dauer der Versuche

ment normal. La gérontologie expérimentale a beaucoup à attendre des recherches, chaque jour plus nombreuses, consacrées aux conséquences lointaines de l'action des radiations ionisantes sur l'organisme.

References

1. *Hardin, B. Jones:* A Special Consideration of the Ageing Process, Disease and Life Expectancy. US Atomic Energy Commission Report UCRL 3105 issued Nov. 1955
2. *Curtis, H. J.,* and *Ruth Healey:* Effects of Radiation on Ageing Report. Int. Congr. Radiobiology, Stockholm 1956; Proceedings, Oliver and Boyd, London 1957.
3. *Verzár, F.,* and *E. Flückiger:* Lack of Adaptation to Low Oxygen Pressure in Aged Animals. 3rd Congr. Int. Assoc. Gerontology, p. 524, London 1954; Livingstone, Edinburgh 1957.
4. *Verzár, F.,* and *E. Flückiger:* Compensatory Hypertrophy of Kidney and Adrenal in the Life-span of Rats. 3rd Congr. Int. Assoc. Gerontology, p. 139, London 1954; Livingstone, Edinburgh 1957.
5. *Verzár, F.,* and *E. Flückiger:* Symposium on Experimental Gerontology, in Basle 1956.
6. *Verzár-McDougall, J.:* Learning and Memory Tests in Young and Old Rats. 3rd Congr. Int. Assoc. Gerontology, London 1954; p. 247, Livingstone, Edinburgh 1957.
7. *Verzár, F.:* Symposium on Experimental Gerontology, in Basle 1956.
8. *Furth, J., A. C. Upton, K. W. Christenberg, W. H. Benedict* and *J. Moschman:* Some Late Effects in Mice of Ionizing Radiations from an Experimental Nuclear Detonation. Radiology 63, 562, 1954.
9. *Blair, H. A.:* Data Pertaining to Shortening of Life-span by Ionizing Radiation. US Atomic Energy Commission Report UR-442, issued May 1956.
10. *Henshaw, P. S.:* Effects of Repeated Small Doses of X-rays on Blood Picture, Tissue Morphology and Life-span in Mice. J. nat. Cancer Inst. 4, 513, 1944.
11. *Miescher, G., J. Plüss* und *B. Weder:* Die Röntgentelangiektasie als Spätsymptom. Strahlentherapie 94, 223, 1954.
12. *Kunkler, P. B., F. R. Farr* and *R. W. Luxton:* The Limit of Renal Tolerance to X-rays. Brit. J. Radiol 25, 190, 1952.
13. *Earlam, M. S.,* and *A. Bollinger:* Experimental Renal Disease Produced by X-rays. J. Path. Bact. 43, 603, 1931.
14. *Bennett, L. R., S. M. Chastain, J. S. Flint, R. A. Manson* and *A. E. Lewis:* Late Effects of Roentgen Irradiation. Radiology 61, 411, 1953.
15. *Minaev, P. F.:* Location Action of X-rays on Various Divisions of the Nervous System of Animals. Conference of the Academy of Science of the USSR on the peacefull uses of atomic energy 1955. Publ. in English. Translation USAE Commission.
16. *Boden, J.:* Radiation Nyelitis of the Brain Stem. J. Fac. Radiol. 2, 79, 1950.
17. *Russell, D. S., C. W. Wilson* and *K. J. Tansley:* Experimental Radionecrosis of Brain in Rabbits. J. Neurol. (Neurosurg.) Psychiat. 12, 187, 1949.
18. *Kohn, H. I.,* and *R. F. Kaltman:* The Effect of Fractionated X-ray Dosage upon the Mouse Testis J. nat Cancer Inst. 15, 891, 1955
19. *Metcalf, R. G.,* and *F. A. Inda:* Chapter 12, Pathology in Animals Subjected to Repeated Daily Exposures of X-rays. Biological Effects of External Radiation; McGraw-Hill, London 1954.
20. *Lorenz, E., W. E. Heston, A. B. Eschenbrenner* and *M. K. Deringer:* Biological Studies in the Tolerance Range. Radiology 49, 269, 1947.
21. *Eschenbrenner, A. B.,* and *E. Miller:* Chapter 5, Effects of Long Continued Total Body Gamma Irradiation in Mice, Guinea-pigs and Rabbits in Pathological Observations in Biological Effects of External X- and γ-radiation. McGraw-Hill, New York 1954.

Effect of Oxygen on Inactivation of Trypsin by the Direct Action of Electrons and Alpha-Particles[1]

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INTRODUCTION

There is abundant evidence that chemical changes produced by the free radicals formed when water is irradiated with radiations of low specific ionization are often different in the presence of dissolved oxygen from those produced in its absence. Although there are a number of possibilities for oxygen to take part in these reactions, the view is widely held that one of the most important factors is the formation of HO_2 radicals (see Gray, *1*, and Alexander, *2*). Changes which *follow on absorption of energy within the molecule which is being studied—direct action*—were believed not to be influenced by the presence of external factors, and when an oxygen effect was found in a biological system indirect action was said to be predominant. Experiments with synthetic high polymers had shown that *direct action could be influenced by outside factors (3); for example, protection by* added substances was shown to occur by energy transfer (*4*), and the over-all yield to increase with increasing temperature (*5*). Similarly, oxygen was found to influence profoundly some of the changes which occur when many proteins (*6*) *and polymers (3, 7) are irradiated, and the most striking effect was to induce* breakage of mainchains in polymers which crosslink in its absence. All these experiments suggest caution in interpreting the existence of an oxygen effect as proof for indirect action in biological systems.

The possibility remains, however, that although oxygen may influence the eventual chemical change it does not affect the total number of molecules which have been damaged by direct action in such a way as to lose their biological activity, and an effect of oxygen on loss of biological activity as a result of direct

22. Dunlop, C. E · The Effect of Roentgen Rays and Exposure to Radium on Fertility. Hum Fert. 12, 33, 1947.

23. van Bekkum, D W.: Phosphorylating Activity of Mitochondria after Total Body Irradiation. Symposium of Radiobiology 1954. edtd. Z. M. Bacq and P. Alexander, p. 201, Butterworth, London 1955.

24 Conard, R. A · Some Effects of Ionizing Radiation on the Physiology of the Gastro-intestinal Tract. Rad. Research 5, 167, 1956.

25. Brauer, R. W, J. S. Krebs and R. Pessotti: The Non-recouperable Fraction of X-irra-diation Injury. US Report.

26. Hagen, C W., and E L. Simmons· Effect of Total Body Irradiation in Rats. Part 1. USNRDL Reviews and Lectures No. 5, September 1955.

27. Kaplan, H S., and M. B. Brown· Mortality of Mice after Total Body Irradiation as Influenced by Alterations in Total Dose, Fractionation and Periodicity of treatment. J. nat. Cancer Inst. 12, 765, 1952.

28. Patterson, E., C. W. Gilbert and J. Matthews: Time Intensity Factors and Whole Body Irradiation. Brit. J. Radiol. 25, 427, 1952.

29. Blair, H. A. · A Formulation of the Injury, Life-span and Dose Relation for Ionizing Radiations. I. Application to the Mouse. UR 206, May 1952.

30. Smith, E. H.: private communication.

31. Carter, R. E., F. P. Bond and P. H. Seymour: The Relative Effectiveness of Fast Neutrons in Mice. Rad. Research 4, 413, 1956.

32. Hursh, J. B , and G. W Casarett: The Lethal Effect of Acute X-irradiation in Rats as a Function of Age. Brit J. Radiol. 29, 169, 1956

33. Kohn, H. I , and R. F. Kallmann: Age, Growth and Lethal X-ray Dose. Science 124, 1078, 1956.

34. Oppenheimer, B. S., E. T. Oppenheimer, I. Danishefsky, A. P. Stout and F. R. Ehrich: Fur-ther Studies of Polymers as Carcinogenic Agents in Animals. Cancer Research 15, 333, 1955.

35. Bacq, Z. M., and P. Alexander: Fundamentals of Radiobiology. Butterworth, Lon-don 1955.

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The possibility remains, however, that although oxygen may influence the eventual chemical change it does not affect the total number of molecules which have been damaged by direct action in such a way as to lose their biological activity, and an effect of oxygen on loss of biological activity as a result of direct

(296)

action must be demonstrated before it can be dismissed as a decisive test for indirect action. Also in radiobiology an oxygen effect is most marked with sparsely ionizing radiations, and on the basis of radical interaction the failure to observe an oxygen effect with, for example, α-rays from polonium or radon is readily understood when the action is indirect. It remains to be shown that the direct action of densely ionizing radiations is influenced less by oxygen than that of sparsely ionizing radiations. To test both these points the inactivation of solid trypsin by 2-Mev electrons, γ-rays from Co⁶⁰, and α-rays from polonium was studied in the absence and presence of oxygen.

EXPERIMENTAL PROCEDURE

Crystallized trypsin having an activity of 2740 units/mg was obtained from Messrs. Armour Laboratories Ltd. and used directly without further purification. The moisture content of our samples was between 4 and 6 %, and the material was irradiated as such, since trial experiments had shown that intensive drying under vacuum did not measurably influence the radiosensitivity.

From 50 to 100 mg of the protein was irradiated in glass tubes sealed under air or vacuum. The protein was spread out as a thin film along the length of the tube so as to facilitate access of oxygen during the irradiation with either 2-Mev electrons from a Van de Graaff generator or γ-rays from Co⁶⁰. In the electron irradiations the samples were passed repeatedly through the beam in such a way that at each pr ss, which lasted approximately 1 second, 10⁵ rads was deposited; an interval of several minutes was allowed to elapse between passes so as to avoid heating the specimen. The γ-irradiation were carried out in a 400-c source at a dose rate of 1100 rads/min. Ferrous sulfate was used for dosimetry, a G value of 16 0 being chosen. There is no detectable effect due to reaction of the protein with ozone formed by irradiation of air surrounding the specimen, since exactly the same inactivation was obtained when samples ranging from 5 to 100 mg of protein were irradiated in tubes of the same size. Also, if ozone produced in this way played a part, this effect would be particularly pronounced when thin layers are exposed to α-rays from a polonium source 0.6 cm away, yet under these conditions there is no oxygen effect.

The irradiation with α-rays was carried out with an external source of 100 mc of polonium electroplated on a platinum disk. The polonium was deposited as a circle of radius 0.8 cm and covered with a mica window. The source was supplied by the Radiochemical Centre, Amersham. The dose delivered to a thin film was determined by measuring the difference between the amount of ferrous sulfate oxidized when the solution was irradiated directly and when covered by polymer film ranging from 6 to 20 μ. The difference between the two values represented the energy retained in the film. The G value used for the α-particles from polonium was 5.9 and was based on the measurements of Miller and Wilkinson (8). For irradiation thin films of trypsin were prepared by evaporation of an aqueous solu-

tion on a microscope cover slip and placed under the α-particle source for different times. For irradiation in the absence of oxygen, carefully purified nitrogen was blown through the irradiation unit both before and during the exposure. The dose rate was of the order of 2.5×10^5 rads/min but varied slightly from experiment to experiment, since the activity of the source decreased during the experiments because the half-life of polonium is only 137 days.

The activity of the irradiated enzymes was determined colorimetrically with casein as a substrate, by the method of Anson (9). Each sample was assayed in duplicate runs at three concentrations and compared with an unirradiated control in the same experiment. The reproducibility between independent experiments was ±5% except when the activity was reduced to less than 30%, when the errors became greater. The irradiated samples remained completely soluble, and in this respect trypsin behaves entirely differently from serum albumin which is rendered insoluble at the isoelectric point with doses greater than 8×10^5 rads (6, 10).
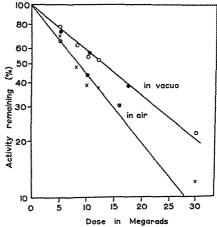
Fig. 1. Relationship between loss of activity and radiation dose for solid trypsin irradiated in air and under vacuum. $\times$ = 2-Mev electrons in air; O = 2-Mev electrons *in vacuo*; $\otimes$ = Co^{60} γ-rays in air; $\bullet$ = Co^{60} γ-rays *in vacuo*.

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recently by Alper and Howard-Flanders (*12*). Simply as an illustration, the two processes might be formulated as follows:

$$
\begin{array}{c}
\text{—CH·CO·NH—} \\
\quad | \\
\quad \text{R}
\end{array}
\xrightarrow{\text{irradiation}}
\begin{array}{c}
\text{—C} = \text{CO} + \text{NH}_2\text{—} \\
\quad | \\
\quad \text{R}
\end{array}
\tag{1}
$$

Peptide chain   Rupture of main chain producing immediate inactivation

$$
\begin{array}{c}
\text{—CH·CO·NH—} \\
\quad | \\
\quad \text{R}
\end{array}
\underset{\substack{\text{reversible in} \\ \text{absence of} \\ \text{oxygen}}}{\overset{\text{irradiation}}{\rightleftharpoons}}
\begin{array}{c}
\text{—C—CO·NH—} + \text{H}' \\
\quad | \\
\quad \text{R} \\
\quad | \quad \text{Free radical} \\
\quad \downarrow +O_2 \\
\quad O_2 \\
\quad | \\
\text{—C—CO·NH—} \\
\quad | \\
\quad \text{R}
\end{array}
\tag{2}
$$

Decomposes

Our experimental observation that peroxide-like free radicals are produced when dry serum albumin is irradiated (*6*) might be considered as support of this hypothesis were it not that there is no perceptible after-effect in the inactivation of trypsin. *The activity is the same when measured within hours of the irradiation as after several days.* On this mechanism the peroxide formed would have to decompose within an hour. The peroxides found in serum albumin, however, are remarkable for their stability. Also, a mechanism of this type has been shown to occur in polymers (*3*), but here the free radicals interact with one another to form cross-links in the absence of oxygen and do not reconstitute.

The alternative mechanism involves the formation of O_2^- radicals. The low-energy secondary electrons which accompany the positive ion, which are formed immediately the protein is irradiated, are captured by the molecule or group in a molecule which has the highest affinity for electrons. Although accurate data are not available for electron affinity of organic materials, it is known that the value for oxygen is extremely high, and there is evidence that O_2^- radicals are formed when polyethylene is irradiated in air (*7*). The O_2^- radical is very reactive and may well inactivate the protein molecule. In the absence of oxygen the secondary electron would be captured by the protein with the formation of a negative ion but this need not produce any far-reaching chemical changes of the kind that are bound to occur when a positive ion is formed.

At present it is impossible to distinguish decisively between the two possibilities,

RESULTS AND DISCUSSION

The effect of oxygen. Figure 1 shows that there is an accurate exponential relationship between radiation dose and loss of activity, and this indicates that inactivation of the enzyme molecule requires a single reaction only. There is, however, a marked influence of oxygen which reduces the inactivation dose by 30% without affecting the exponential relationship. On the other hand, for irradiations with α-particles there is no detectable oxygen effect and there is again an accurate linear relationship between dose and percentage inactivation (see Fig. 2).

There would appear to be two ways by which oxygen can enhance enzyme inactivation by direct action (i.e., when free radicles formed in water are not involved). For the first mechanism one would have to assume that radiation produces two types of chemical change in the protein, only one of which brings with it immediate inactivation. The other is only potentially capable of inactivating the molecule after combination with oxygen. This second reaction could be the formation of a fairly unreactive free radical which in the absence of oxygen reverts back to its original state without causing any inactivation. In the presence of oxygen it reacts to a peroxide which is inherently unstable and decomposes spontaneously in such a way as to bring about inactivation. The possibility that the oxygen effect *in vivo* might occur in this way was first suggested by Bacq and Alexander (*11*) and more
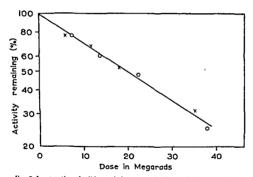
Fig 2. Inactivation of solid trypsin by α-particles from polonium O = in air;
X = under nitrogen.

since energy transfer processes occur both between and within molecules in the solid state (3, 4). Also, direct analytical data have shown that the number of chemical reactions occurring in organic substances cannot be correlated with the number of so-called "primary" ionizations. For example, in polyisobutylene (5) one interruption in the main chain occurs at random for every 10-ev set of energy taken up by the system, although according to the target theory 100 ev would be assumed to be necessary. In fact, earlier work by Lea *et al.* (16), which has recently been confirmed by Deering (17), had shown that the target theory—the value of 100 ev per "primary ionization" for the inactivation of the small enzyme ribonuclease—gave a value for the molecular weight which differed from that established unambiguously by analytical methods (18) by more than 100%. Biochemical considerations also indicate that one event anywhere within the molecule is unlikely to bring about inactivation every time since there is convincing evidence that many of the side chains of the protein can be severely altered without loss of activity. The target size should therefore be smaller than the molecular size, yet the opposite is found in these experiments. The existence of energy transfer processes, and other reactions such as the oxygen effect, when solids are irradiated, make it impossible to adopt the purely statistical approach inherent in the target theory for the calculation of sensitive volume. But probably these factors are most serious when the sensitive volume is relatively small as in the case of trypsin, and for much larger structures such as viruses the values deduced from target calculations will be more informative.

SUMMARY

1. The radiation dose required to inactivate solid trypsin with 2-Mev electrons and Co^{60} γ-rays is 30% greater in the absence than in the presence of oxygen. No oxygen effect is found for α-irradiations.

2. Possible mechanisms for an oxygen effect when the action is direct are discussed. The existence of an oxygen effect in an *in vivo* system cannot be used as a test to establish that the radiation acts predominantly through the indirect action of free radicals formed in water.

3. The sensitive volume for trypsin calculated from conventional target theory is much greater than the molecular size, and the value obtained depends on the condition of the irradiation.

4. The limitation of the target theory for determining the size of relatively small molecules are discussed in the light of recent radiochemical experiments, and reasons are advanced why it does not apply to small enzymes.

ACKNOWLEDGMENTS

but these experiments serve to emphasize that even when the action is direct there is a pronounced oxygen effect for the inactivation of enzymes by sparsely ionizing radiations and that this behavior is not necessarily characteristic for indirect action.

The absence of an oxygen effect with α-irradiation would be expected in either of the two mechanisms discussed, since in the vicinity of the α-track the number of ionizations is so great that the proportion of molecules inactivated cannot be increased by the introduction of additional processes so long as these occur within the range of the δ-rays from the track.

"*Target size.*" According to the theoretical treatment developed by Lea (*13*) and also used by Pollard *et al.* (*14*) for computing target size from radiation data, an enzyme is inactivated every time a primary ionization occurs within its sensitive volume. From Wilson cloud chamber photographs and other data it is known that in gases ionization occurs in clusters with an average size of three ion pairs. Since each ion pair requires an expenditure of energy of between 30 and 35 ev, one primary ionization (i.e., a cluster of three ion pairs) occurs for every 100 ev of energy deposited in the gas by the irradiation. Lea, having no evidence to the contrary, used this value also for primary ionization in solids for which this information cannot be obtained experimentally. With this assumption it is possible to calculate a target volume from the radiation dose required to produce a given loss of activity. The whole basis of such a calculation depends on the assumption that inactivation follows immediately on the primary ionization and that it cannot be influenced by external factors. This concept is difficult to reconcile with an oxygen effect such as described in this paper. Pollard *et al.* (*14*) had reported experiments on the inactivation of dry trypsin, but these were confined entirely to irradiation *in vacuo.* They obtained within experimental error the same value for the "target size" and the molecular weight of trypsin as determined by physicochemical methods. The data in this paper do not bear out the agreement found by Pollard *et al.* (*14*); the "target size" calculated (according to the associated volume method of Lea, *13*) from the inactivation data shown in Figs 1 and 2 are listed in Table I.

There is clearly no close quantitative relationship between "target size" and molecular dimensions. This is in accord with radiochemical experiments which suggest that molecular weights cannot be obtained from so-called target-size data,

TABLE I

"TARGET SIZE" FOR TRYPSIN FOR DIFFERENT IRRADIATIONS

Irradiation	$D_c{}^a$	Molecular weight from target theory
2 Mev and γ-rays in vacuo	18 4 × 10⁶ rads	4 2 × 10⁴
2 Mev and γ-rays in air	11 8 × 10⁶ rads	6 6 × 10⁴
α-rays (nitrogen and air)	28 × 10⁶ rads	9 0 × 10⁴
Molecular weight by physicochemical methods (*15*) = 2.3 × 10⁴		

ᵃ Dose required to reduce the activity to 37% of that before irradiation

Effect of X-Rays and γ-Rays on Synthetic Polymers in Aqueous Solution

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INTRODUCTION

In recent years, considerable research has been devoted to the changes produced in polymers when these are irradiated in the solid state. The major effects observed can be ascribed to the formation of crosslinks or to the fracture of the main chain, depending on the particular polymer studied.[1] Other changes of a less striking character, such as differences in unsaturation and fracture of side chains, are also found.[2]

A separate set of investigations intended for the interpretation of radiobiological phenomena has been carried out on the irradiation of aqueous solutions of long chain polymers.[3] In this case, the changes produced in the polymer can be of a more complex character and may result either from *direct* action where the energy from the incident radiation is captured directly by the polymer molecule or from *indirect* action where the energy captured by the water molecules causes chemical changes in these molecules and the products formed subsequently react with and modify the polymer molecules. The amount of energy taken up on exposure to ionizing radiation (such as fast electrons, x-rays, or gamma rays) depends almost entirely on the mass of the irradiated material and is practically independent of its chemical composition. In irradiated polymer solutions the solute and the water therefore absorb approximately equal amounts of energy on a weight basis and the fraction of the total absorbed energy which is taken up by the polymer directly is proportional to its concentration. Thus the average energy absorbed *directly* per polymer molecule is independent of concentration. This is not true for the *indirect* effect.

Distinction between direct and indirect is important in radiobiological studies and several methods may help to differentiate between these two processes, although their interpretation may not be conclusive. Additives or protectors added to the solution may react with the radiation products of the water and thereby protect the polymer molecule against the indirect effect, although protection can also occur in cases in which the action has been direct.[4] The decisive test is the influence of concentration. *Where*

REFERENCES

1. L H GRAY, A. D. CONGER, M. EBERT, S HORNSEY, and O. C. A. SCOTT, The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Brit. J. Radiol.* **26**, 638-648 (1953).

2. P. ALEXANDER, A physico-chemical method of testing the protective action of chemical compounds against the lethal effects of ionizing radiations. *Brit. J. Radiol.* **26**, 413-416 (1953).

3 P ALEXANDER and A. CHARLESBY, Physico-chemical methods of protecting against ionizing radiations In *Radiobiology Symposium 1954*, pp. 49-60, Butterworth, London, 1956.

4. P ALEXANDER and A. CHARLESBY, Energy transfer in macromolecules exposed to ionizing radiation. *Nature* **173**, 578-580 (1954).

5 P. ALEXANDER, R. M. BLACK, and A. CHARLESBY, Radiation induced changes in the structure of polyisobutylene. *Proc. Roy. Soc* **A232**, 31-48 (1955).

6 P. ALEXANDER, M FOX, K. A. STACEY, and D. ROSEN, Comparison of some direct and indirect effects of ionizing radiations in protein. *Nature* **178**, 846-849 (1956).

7. P. ALEXANDER and D. TOMS, The effect of oxygen on the changes produced by ionizing radiations in polymers *J. Polymer Sci.* **12**, 343-348 (1956).

8. N MILLER and J. WILKINSON, The oxidation of ferrous sulphate by α-particles *Trans. Faraday Soc.*, **50**, 690-699 (1954).

9. J H NORTHROP, M. KUNITZ, and R. M HERRIOTT, *Crystalline Enzymes*, Columbia University Press, New York, 1948

10. R. SETLOW, Radiation studies of proteins and enzymes. *Ann. N. Y. Acad. Sci.* **59**, 471-483 (1955).

11 Z. M. BACQ and P. ALEXANDER, *Fundamentals of Radiobiology*, p. 66, Butterworth, London, 1955.

12. T. ALPER and P. HOWARD-FLANDERS, Role of oxygen in modifying the radiosensitivity of *E. coli. Nature* **178**, 978-979 (1956).

13. D. E. LEA, *Actions of Radiations on Living Cells*, Cambridge University Press, Cambridge, 1946

14 E. POLLARD, A. BUZZELL, C. JEFFREYS, and F. FARRO, Inactivation of pepsin and trypsin by deutron and electron bombardment *Arch. Biochem and Biophys.* **33**, 9-21 (1951).

15 H NEURATH and W. J. DRYER, Mechanism of activation of trypsinogen and chymotrypsinogen. *Discussions Faraday Soc* **20**, 32-43 (1955)

16. D. E LEA, K. M SMITH, B. HOLMES, and R. MARKHAM, Direct and indirect action of radiation on viruses and enzymes *Parasitology* **36**, 110-118 (1944)

17. R. A. DEERING, Inactivation cross sections of dried invertase and ribonuclease as a function of LET. *Radiation Research* **5**, 238-246 (1956).

18. S. MOORE, C. H W HIRS, and W H. STEIN, Studies on structure of ribonuclease. *Federation Proc* **15**, 840-848 (1956).

dialyzed against distilled water till free from monomer. Alternatively 0.015% persulfate and 1.5% isopropyl alcohol were added to the 10% solution, which was then deoxygenated and raised to 60°C. for two hours. Residual monomer and catalyst were removed by dialysis. Polymethacrylic acid set to a gel after irradiation and this was dissolved in alcohol and then dialyzed against water.

Polystyrene sulfonate was prepared by treatment of polystyrene with a mixture of concentrated and fuming sulfuric acid. Samples of polyvinyl and of polyvinyl pyrrolidone were kindly provided by Messrs. I.C.I. and May & Baker. Analysis of the polyvinyl alcohol obtained from I.C.I. failed to reveal the presence of any acetyl groups. The molecular weight as determined by light scattering was found to be 1.29×10^5 but increased on standing due to aggregation.[6]

Viscosity Determination

The polymers obtained by x-irradiation and the polystyrene sulfonate were of high molecular weight and their solutions were markedly non-Newtonian. Their viscosities were determined at 20°C. in a flatbed viscometer[7] at a number of shear rates. Values reported refer to a shear rate (β) of 50 sec.$^{-1}$ at one concentration since extrapolation to zero concentration is very difficult under these conditions.[7] The polymers prepared with the catalyst were often of lower molecular weight and their intrinsic viscosities $[\eta]$ expressed in g./100 ml. were obtained from measurements at a number of concentrations in an Ubbelohde viscometer at 25°C. The changes in viscosity referred to below are expressed in percentages as:

$$\frac{\text{(viscosity before irradiation)} - \text{(viscosity after irradiation)}}{\text{viscosity before irradiation}} \times 100$$

Changes in the weight-average molecular weight of polymethacrylic acid after irradiation were determined by Dr. K. A. Stacey by light scattering as described earlier.[8]

Irradiation Technique

For low doses 140 kv. x-rays filtered by copper were employed and for higher doses the γ-rays from a 400 curie cobalt 60 source in a position where the dose rate was 1000 r./min. Solutions were freed from oxygen by prolonged bubbling of nitrogen which had been carefully freed from oxygen by passing over red hot copper and through a solution of sodium hydrosulfite and anthraquinone sulfonate.

The yield of radiochemical reactions is expressed as the number of reactions occurring for every 100 e.v. of energy deposited in the system by the radiations. This is known as the G value; it does not imply that the 100 e.v. is used exclusively in the reaction referred to. For example, when irradiating a polymer, there will be one G value for each type of change in the main chain, and one for the formation of each of the different gases evolved.

the radiation effect is direct, *i.e.*, where the energy is initially taken up by the polymer molecules, there should be no change with concentration. (There may be an increased efficiency of crosslinking at higher concentrations, as the polymer molecules are closer together.) If the effect is indirect, the higher concentration of polymer will be less affected by a given radiation dose, there being more polymer molecules competing for the activated water products. The purpose of this work was to study the effect of polymer concentration on the changes produced by radiation and hence to derive information on the mechanism of direct and indirect action.

Radiolysis of Water

In its passage through matter, ionizing radiation (*e.g.*, fast electrons, x-rays and γ-rays, fast protons) produces ionized and excited atoms and free electrons. These products may then interact with one another and with neighboring molecules to produce free radicals and initiate chemical changes. These effects have been determined in detail in only a few cases, since the mechanism is understood only in general terms and it is not possible to predict the products of radiolysis except perhaps within a closely related series of organic compounds.

By far the greatest amount of research has been done on aqueous systems and chemical changes produced on irradiating water are reasonably well understood, although many points of detail remain to be elucidated.[3] In the absence of oxygen, water is decomposed into OH and probably H radicals (although there is no direct evidence for the formation of the latter), hydrogen gas and hydrogen peroxide also being formed. These molecular products can interact with the radicals to re-form water; consequently, the concentration of H_2 and H_2O_2 produced is affected by the presence of dissolved substances which can act as scavengers for the radicals. In pure (*i.e.*, oxygen-free) water where all the radicals are available for reaction with the molecular products, the equilibrium concentration of H_2 and H_2O_2 produced by x-rays is too low to be detected. The distribution of the radicals formed will depend on their concentration and relative rates of diffusion and with densely ionizing radiation such as α-particles molecular products may build up even in pure water. Hydrogen atoms react very rapidly with oxygen to give HO_2 radicals, which can then react with the solute. Consequently, radiochemical reactions are frequently entirely different in aerobic than in anaerobic conditions.

EXPERIMENTAL

Preparation of Polymers

Samples of acrylic acid, methacrylic acid, and acrylamide were polymerized in aqueous solution by irradiation with 140 kv. x-rays or by using per sulfate as a catalyst. A 10% solution of monomer was completely deoxygenated (see below) and irradiated with a dose of 10^4 r. at 324 r./min. Immediately after the irradiation, air was admitted and the solution
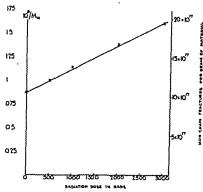
Fig. 1. Molecular weight changes in irradiated polymeth-
acrylic acid. 0.355% solution, ionized, x-ray irradiation, molecular
weight changes from light scattering. (The number of main chain
fractures is expressed as the number necessary to reduce a polymer
of infinite weight to one having the M_w used in the reciprocal on
the left axis.)

fracture of the main chain, rather than to linking between monomers in the
molecule resulting in a smaller swept volume, some absolute determinations
using light scattering were carried out. 20 ml. of a 0.355% solution of
polymethacrylic acid (60% neutralized) was irradiated with 140 k.v. x-rays
and the molecular weight determined by light scattering. This was carried
out at a number of different doses and the results plotted in Figure 1.
These determinations give the weight-average molecular weights; for a
random molecular weight distribution there are twice the number averages,
which are inversely proportional to the number of chain fractures. Figure
1 shows that the reciprocal weight-average molecular weight, and hence
the number of main chain fractures,[2] increases linearly with radiation dose.
The G value for main chain fracture deduced from this plot is 1.7, in good
agreement with the value (1.6) obtained by Alexander and Fox[2] using
viscosity measurements.

In the absence of oxygen, very little degradation is observed (see Table I)
except when the doses are so high that sufficient hydrogen peroxide is pro-
duced as a "molecular product" to be equivalent to the amount of oxygen
dissolved in aerated solutions. The accumulation of hydrogen peroxide
makes it very difficult to maintain truly oxygen-free conditions when
relatively high doses have to be used, and for this reason we have preferred
to study high molecular weight polymers which show significant changes at
low doses when the quantity of hydrogen peroxide produced can be neg-
lected.

Gel Formation

After irradiation, many polymers become crosslinked into a gel structure which is swollen in the initial solvent. The transformation from a viscous fluid to a lightly crosslinked gel can be determined by visual inspection of the irradiated specimens.

BEHAVIOR OF DILUTE SOLUTIONS

Degradation of Polymethacrylic Acid

The first system to be studied was polymethacrylic acid[3] in the concentration range 0.01 to 0.1%, when it could be assumed that the changes observed would be due entirely to indirect action. The effect of x-rays was to degrade the molecule (*i.e.*, to reduce the average molecular weight by main chain fracture). In comparative experiments the degradation could be conveniently followed by determining the decrease in viscosity after irradiation measured at a standard concentration. The magnitude of the effect observed depends on the concentration and initial molecular weight of polymer as well as on the radiation doses.[3,9]

Some results for a series of polymethacrylic acid solutions irradiated at various dilute concentrations and for varying doses are shown in Table I.

TABLE I

DEGRADATION OF POLYMETHACRYLIC ACID BY X-RAYS

(Initial $M_w = 2.1 \times 10^4$)

Concentration	Ionization degree (α)[a]	Condition	Dose	Decrease in viscosity (%)[b]
0 025%	0 0	In air	1000 r.	45
"	"	In N$_2$	"	4
	0 6	In air	"	67
0 05%	"	In air	2000 r.	62
0.1%	"	In air	4000 r.	64
0 025%	"	In N$_2$	1000 r.	3
	"	In N$_2$	10^4 r.	16
	"	In N$_2$	10^5 r.	85

[a] Denotes the degree of ionization *i.e.* the fraction of —COOH groups neutralized by [...] tion is 7.1.
[...] 5% polymethacrylic acid; α =

The doses and concentrations are so low that only indirect action can play any part in the observed changes. In the *presence of air*, the radiation dose needed to produce a given decrease in viscosity (*i.e.*, the same number of main chain fractures per molecule) is directly proportional to concentration. In other words, a given radiation dose will produce the same *total* number of main chain fractures in the polymer molecules independent of the concentration of these molecules over the range considered. This behavior is typical of the indirect effect.

To confirm that the observed decreases in viscosity are in fact due to a

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to study high molecular weigh
low doses when the quantity
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Since oxygen must be present for degradation polymethacrylic acid, we were led to the conclusion that the main chains were broken by HO_2 radicals and that H and OH were ineffective for this purpose. An alternative mechanism, in which oxygen is required for production on the polymer of peroxides which subsequently decompose with main chain breakdown, was rejected in the case of polymethacrylic acid.[8,9] Wall and Magat[10] found oxygen to be necessary for the degradation of polymethyl methacrylate and polystyrene when dissolved in chloroform and the mechanism probably differs from that in aqueous solutions since HO_2 radicals cannot be formed.

When, more recently, we repeated these earlier experiments with polymethacrylic acid, only small differences between deoxygenated and aerated solutions were found; degradation was observed in completely oxygen free solutions (see Table II). This unexpected behavior was traced to a small change in the method of preparation of the polymer. After x-ray polymerization in 10% solution, the polymethacrylic acid sets to a gel; in the earlier experiments, this gel was stirred in water kept at 70–80°C. until it dissolved, a process taking several days. In the later experiments, the gel was dissolved rapidly at room temperature in ethyl alcohol, which was removed by dialysis. We found[11] that during the polymerization with x-rays peroxide groups were introduced into the polymer although the solution was quite free from oxygen. Heating of the polymer destroys the peroxide groups and makes the polymer resistant to irradiation in the absence of oxygen (see Table II). It would appear that the peroxide groups represent points of weakness at which rupture of the main chain can be produced by OH or H radicals in the absence of dissolved oxygen.

TABLE II

OXYGEN EFFECT IN DEGRADATION OF POLYMETHACRYLIC ACID BEFORE AND AFTER HEATING TO REMOVE PEROXIDE GROUPS

(Irradiated at 0.025% concentration, 60% ionized, and irradiated with 1000 r.)

Treatment of polymer	Radiation condition	Decrease in viscosity (%)*
None	Air	63
	N_2	40 9
0.1% soln. heated under air at 90°C. for 48 hrs.	Air	58
	N_2	10 5
0.1% soln. heated in vacuo at 90°C for 48 hrs.	Air	54 3
	N_2	5.7

* As in Table I.

Degradation of Polyacrylic Acid, Polyacrylamide, Polystyrene Sulfonates, Polyvinylpyrrolidone, and Polyvinyl Alcohol

Preliminary experiments[1] indicated that other water-soluble vinyl polymers, such as polyacrylic acid, were degraded in dilute solutions. An inorganic polymer sodium polyphosphate:

the ionized form) is not degraded by radiation; in aerated solutions the acid form is somewhat more resistant to radiation. Likewise ionized polyacrylic acid does not degrade in the absence of oxygen. Unionized polyacrylic acid, however, does degrade at nearly the same rate as in air (Table III, Fig. 2).

Polyacrylamide behaves like polyacrylic acid in unionized form—it degrades significantly in the absence of oxygen although less than in air. The results for two samples of different molecular weight are shown in Table IV. The higher molecular weight samples show significant changes in viscosity at much lower radiation doses (see Fig. 3). This difference is not due to any inherent change in sensitivity but arises from the fact that the same number of main chain fractures in a given weight of polymer produces a greater relative change in molecular weight in a high molecular weight material.

Polystyrene sulfonate shows no oxygen effect at all being equally readily degraded in aerated solution or under nitrogen (see Table V).

TABLE V

DEGRADATION OF POLYSTYRENE SULFONATE BY X-RAYS

Concentration		Condition	Dose	Viscosity decrease* (%)
0 015%	..	Air	1000 r.	44.7
		N₂	"	43 6
		Air	200 r.	57 3
		N₂	"	54 8

* Measured at 0 015% concentration in Ubbelohde viscometer. Specific viscosity before irradiation 1.15. In the absence of added salt, no extrapolation to $c = 0$ is possible because of the polyelectrolyte nature of the solution.

The position with regard to polyvinyl alcohol and polyvinylpyrrolidone is less clear. When irradiated in 0.1% solution, the viscosity of both polymers is decreased more in the presence than in the absence of oxygen. The molecular weights of the polymers studied were however low (light scattering gave 1.3×10^5 for the sample of polyvinyl alcohol used.[6] To produce measurable changes in each molecule, correspondingly high doses of radiation were necessary which introduced the complication of hydrogen peroxide formation. In any case, with these polymers, it is difficult to relate viscosity to molecular weight since they aggregate in solution.[6]

These experiments show that all the vinyl polymers examined when irradiated in dilute solution suffer main chain fracture and consequent degradation; the breaking of the C—C bond must be the result of reaction with free radicals formed in the water. A common mechanism does not, however, appear to be operative since the influence of oxygen is different in different polymers. A possible interpretation is that reaction with OH radicals can break main chains in only some of the polymers examined and that the others can be fractured by HO₂ radicals.

TABLE IV

DEGRADATION OF POLYACRYLAMIDE BY X-RAYS

Concentration	Irradiation dose	Condition	Intrinsic viscosity
High molecular weight			8 0
0.0625..	⸱ 500 r.	Air	7 26
	1000 r.	Air	6 0
	2000 r.	Air	4 5
	2000 r.	Nitrogen	6.0
Lower molecular weight			2.7
0 259%.	10,000	Air	2.39
	20,000	"	2 00
	30,000	"	1 90
	10,000	Nitrogen	2 52

eliminate the possibility of peroxide groups introducing spurious results, all these polymers were heated in carefully deaerated solution at 100°C. for 45 hrs., a treatment which was more than sufficient to destroy these groups (see Table II).

In the absence of oxygen, polymethacrylic acid (either in the acid or in
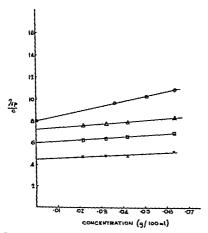
Fig. 3. Degradation of polyacrylamide. 0 0625% solution irradiated under air by x-rays. (X) = 2000 r. (□) = 1000 r. (△) = 500 r. (O) = 0 r.

further to very large volumes when placed into excess water after irradiation. As the irradiation is increased, so the swelling of the gel becomes progressively less until, at a dose depending on the polymer and its original concentration, the gel pulls away from the walls of the vessel and floats freely in a solution which is essentially free from polymer (see Fig. 4).

Fig. 4. Gel formation in irradiated polyvinyl alcohol. (A) Unirradiated. (B) Gelling radiation dose. (C) Highly crosslinked gel shrinking away from walls.

So far we have confined our attention largely to the dose required to produce a gel and have not attempted to interpret the subsequent swelling behavior of the gels formed with higher doses. The general pattern is
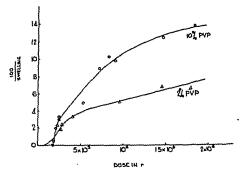
Fig. 5. Increase in crosslinking with concentration and radiation dose. Polyvinylpyrrolidone, γ-radiation at 1000 r./min.

shown in Figures 5 in which crosslinking is expressed as 1/swelling. The rate at which crosslinking proceeds depends on the initial concentration and except in very dilute solutions (i.e., near the changeover point from degradation to crosslinking) increases with increasing concentration.

CROSSLINKING IN SOLUTION

With the exception of polymethacrylic acid, all the water-soluble polymers examined (which degrade in dilute solution) crosslink when irradiated as solids (*i.e.*, when the action of the radiation is direct). It therefore became of interest to examine their behavior in more concentrated solutions where direct action can produce a significant contribution to the over-all effect. Unexpectedly we found that the following polymers when irradiated at concentration as low as 1% (when direct action cannot play an important role) were not degraded by irradiation but became crosslinked: *polyvinyl alcohol, polyvinylpyrrolidone, polyacrylamide, polyacrylic acid* (unionized). However, polyacrylic acid, when partially neutralized with sodium hydroxide, and therefore carrying a charge, degrades at all concentrations; a degree of ionization of 0.1 is already sufficient to inhibit crosslinking. Polymethacrylic acid both ionized and unionized degrades at all concentrations; with solutions containing as much as 10% of polymer the degradation appears to be almost entirely due to indirect action since the amount of change for a given dose is inversely proportional to concentration as at lower concentrations (see section on Behavior of Dilute Solutions).

TABLE VI

CHANGES IN VISCOSITY OF POLYVINYL ALCOHOL AFTER IRRADIATION BY
γ-RAYS

Concentration		Dose	Condition	Intrinsic viscosity
Sample 1:	—	—	—	0 55
	0 3%	1 4 × 10^6 r.	N_2	0 59
	0 3%	1 4 × 10^6 r	Air	0 28
	7%	2 7 × 10^6 r	Air	0 57
	7%	4 1 × 10^6 r.	Air	1 18
	7%	4 3 × 10^6 r.	Air	Sets to gel
Sample 2:	—	—	—	0 67
	0 3%	1 5 × 10^6 r.	N_2	0 70
	1%	0 84 × 10^6 r	Air	2 84
	1%	0 92 × 10^6 r.	Air	gel
	2%	0 90 × 10^6 r.	Air	1 69
	4%	1 2 × 10^6 r.	Air	1 30
	0 3%	1 5 × 10^6 r.	Air	0 40
Sample 3·	—	—	—	0 76
	0 5%	0 9 × 10^6 r	Air	0 76
	0 5%	"	N_2	1 42
	0 5%	1 2 × 10^6 r.	Air	0 77
	0 5%	1 5 × 10^6 r.	Air	0 85
	2%	0 9 × 10^6 r	Air	1 15

The occurrence of crosslinking may be recognized in the polymer solution by the fact that the viscosity does not fall but increases slightly (see Table VI) until at a well-defined dose the solution sets to a swollen gel which swells

acrylamide (see Table VII) but these are sufficient to show that crosslink-
ing in solution follows the same general pattern.

MECHANISM OF CROSSLINKING

Any theory proposed for the behavior of polymers irradiated in solution
must account for the following main observations, applicable to a number of
polymers:

(1) Degradation at low concentrations.
(2) Crosslinking at higher concentrations.
(3) The sharp change from one reaction to the other in a very limited
range of concentration (about 0.4–0.6%).
(4) The increase in the radiation dose for gelling with increasing con-
centration above 1%.

The following suggestions will be considered below:

(1) Degradation is due to indirect action via water molecules, cross-
linking results from direct action.

(2) Internal linkages are formed between monomers in the same poly-
mer molecule. The formation of such internal linkages competes with the
formation of external linkages between separate molecules.

(3) An alternative mechanism of gel formation relaying on main chain
scission to produce a gel. This has been considered theoretically.¹² [This
last value was calculated as follows: the dose necessary to produce a detect-
able amount of insoluble material on irradiating the dry polyacrylic acid
(weight-average molecular weight 2.2×10^5) used in these experiments is
2.3×10^6 r. and this dose deposits 0.14×10^{11} e.v. in 1 g. of solid polymer.
For gel formation to occur a minimum of one crosslinked monomer unit per
weight average molecule is required on the average, so that the energy
absorbed per crosslinked unit is $0.14 \times 10^{11} \times 2.2 \times 10^5 \times 1.66 \times 10^{-24}$ or
52 e.v. There are two crosslinked units per crosslink so that a total energy
of 104 e.v. is absorbed per crosslink formed in dry polyacrylic acid (G
per crosslink = 1).]

(4) Unstable centers are formed in polymer molecules which can either
lead to crosslinking or to degradation depending on the molecular environ-
ment.

(1) **Direct versus Indirect Action.** The hypothesis that degradation
is due to indirect action and crosslinking due to direct action does not fit
the observed facts for the following reasons:

(1) Since the changeover occurs at approximately 0.5% concentration
direct action leading to crosslinking would have to be 200 times as effective
as indirect leading to degradation. We have determined the ratio ex-
perimentally and find that the G value for degradation of polyacrylic acid
in extremely dilute solution is about 1 while that for crosslinking by irradia-
tion of the dry polymer is likewise 1.

On this basis, the lowest concentration at which crosslinking could occur
would be about 50% of polymer but this would almost certainly be an under-
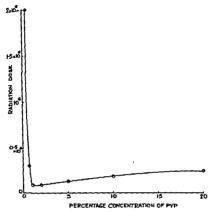
Fig. 6. Radiation dose for gel formation in polyvinylpyrroli-
done solutions.   γ-Radiation at 1000 r./min.

A most remarkable feature of the crosslinking reaction in the very sharp
changeover from degradation to efficient crosslinking. This is illustrated
for polyvinylpyrrolidone in Figure 6; degradation occurs at a polymer
concentration of 0.3% while in a 1% solution the dose required to produce
a gel has reached its minimum value. With polyacrylic acid of molecular
weight 2.2×10^5 the changeover from degradation to crosslinking occurs
at 0.1% concentration and the dose required to produce a gel is at a mini-
mum at a concentration of approximately 1%. This system is now being
studied in detail. We have only obtained preliminary results with poly-

TABLE VII

CROSSLINKING OF POLYACRYLAMIDE[a]

Concentration	Dose	Swelling of gel[b]
1.08%	0.6×10^6 r.	72.5
	1.27×10^6 r.	62
2.3%	0.26×10^6 r.	171
6.7%	1.5×10^6 r.	38.5
15%	0.8×10^6 r.	38.0
30.4%	1.0×10^6 r	42

[a] Intrinsic viscosity of starting material is 2.7.
[b] Weight of water taken up by polymer/weight of polymer.

(3) **Endlinking.** It has generally been accepted that radiation induced linking between molecules and consequent gel formation arises from the fracture of bonds in the side chains, whereas degradation arises from fracture of main chain bonds. An alternative theory termed endlinking has however been investigated theoretically.[14] With this theory, it was shown
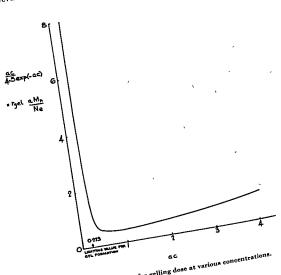
Fig. 7. Theoretical relation for gelling dose at various concentrations.

that the formation of a gel structure could be equally accounted for by main chain fracture, the assumption being made that when a polymer molecule is fractured, the two fragments can attack and link themselves on to neighboring inert polymer molecules. At first, this induces branching and eventually a three-dimensional network may be formed. For these polymers whose fractured ends can become stabilized by a molecular rearrangement, such "endlinking" will occur less frequently and degradation may ensue. This theory therefore implies that the primary radiation act

formed in the solvent, remains in this state for a time during which it can
. . . . polymer molecule. If, during this time, no
. . . .
linking, the activated polymer . . . . t
to stabilize itself in the course of which it suffers main chain fracture and
degrades. The following simplifying assumptions will be made for mathe-
matica . . .

(1) . . . .
mer units in the same molecule.
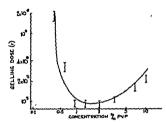
Fig. 8. Gelling curve for polyvinylpyrrolidone: (I)
experimental; — theoretical curve $a = 55$.

(2) The energy required to activate the polymer molecule is the same,
whether the effect be direct or indirect. Where this is not the case, a simple
correction can be readily applied.

(3) The initial molecular weight distribution is of the random (ex-
ponential) type. The nonvalidity of this assumption would only alter the
parameters defining the gelling curve to a minor extent. None of the main
conclusions would be affected.

The probability of an activated monomer unit becoming linked to a
neighboring unit depends on the polymer concentration, but cannot exceed
unity. Fow low concentrations, when mutual exclusion effects are negli-
ble, this probability is proportional to the polymer concentration c. The
constant of proportionality, denoted by a, is related to the time available
for an activated unit before it rearranges itself into a stable degraded prod-
uct. a is independent of concentration and of the molecular weight of the
polymer. Its reciprocal represents the minimum polymer concentration
which, but for exclusion and statistical fluctuations, would ensure that all
activated units result in crosslinks. At very low concentrations, the ratio
$ac/(1 - ac)$ represents the number of crosslinked/degrading units. At
. . . . . . a more complicated expression obtains because of
. . . . leads to the following

may be similar for a range of polymers, but the subsequent effects (crosslinking or degradation) depend on the stability of the ensuing radicals.

Although this mathematical theory of endlinking fits the observed facts as well as the conventional crosslinking theory, no experimental evidence for it has been obtained. The equations derived have been applied by us[13] to account for the concentration effect in polymer solutions. It is assumed that the polymer molecules irradiated in solution suffer fracture of the main chain. The two ends formed are unstable and eventually a molecular rearrangement occurs near the ends in order to stabilize them. This takes a certain time, during which either or both of these active ends may link themselves to other polymer molecules if these exist in the vicinity. The observed effects of main chain fracture will therefore depend on polymer concentration. If this is low, most main chain fractures result in degradation and reduced molecular weight. If it is high, branching and eventually gel formation will occur. It can be shown theoretically[14] that, for an initial random molecular weight distribution, gel formation by endlinking first occurs when there is an average of radiation induced main chain fracture per three molecules, the two fractured ends subsequently linking themselves to adjacent molecules (in other words, there must be at least two active ends per six inactive ones for gel formation). In the case of polymer solutions the number of radiation-induced fractures will be $0.6 \times 10^{14} r/e$ (see section (2) above) and of these a fraction α will result in endlinking. The parameter α will vary with polymer concentration in an unknown but smooth manner. The number of degrading fractures will be $(1 - \alpha) \times 0.6 \times 10^{14} r/e$ and these fractures will give an increased number of separate molecules to be linked together. Then for gel formation, the following equation must be satisfied:

$$0.6 \, \alpha \times 10^{14} \, r/e \geq \text{}^1/_3 \{ (1 - \alpha) \, 0.6 \times 10^{14} \, r/e + cN/M_n \}$$

where cN/M_n is the number of polymer molecules initially present in 1 g. of solution. The minimum gelling radiation dose:

$$r_{gel} = \frac{cNe}{M_n} \frac{0.6 \times 10^{14}}{(4\alpha - 1)}$$

This formula gives the required sudden change from degradation (when $\alpha < 0.25$) to gel formation (when $\alpha > 0.25$). With a feasible variation of α with concentration, the observed effects can be explained.[13] The main objection to this theory arises on chemical grounds, as it is not easy to explain the chemical processes involved which would produce endlinking without a continued radical formation. No direct evidence for or against this theory is available.

(4) **Simultaneous Crosslinking and Degradation.** This theory assumes that a polymer molecule activated either directly, or via radicals

Additives

The effect of radiation on polymer solution, as measured by the tendency to crosslink or degrade, can be profoundly affected by the presence of small amounts of certain additives, which may either reduce the radiation effect or shift its emphasis. Oxygen is one such additive, but a number of others such as thiourea and β-mercaptan have been studied.[13] In solutions such additives may act in one of several ways. They may react with the activated polymer molecule; if the compound formed is a stable one, then the additive has blocked the reactive point and prevented both crosslinking and degradation (i.e., protection will have occurred). It is also possible that the additive combines with the active center to give a structure which can still degrade but which will no longer crosslink. For example, the effect whereby oxygen decreases crosslinking but promotes degradation could arise as follows:

$$RH \xrightarrow{\quad} R^* \xrightarrow{\quad O_2 \quad} RO_2$$

RH	R*	RO₂
polymer	active center which can degrade or crosslink	peroxide which can no longer crosslink, but may still degrade

(radiation)

A detailed study of the effects produced by a number of additives will be published later.

References

1. Charlesby, A., *Proc. Roy. Soc. London*, 1952, 215, 187; *Nature*, 1953, 171, 167. Lawton, E. J., Balwit, J. S., and Bueche, A. M., *ibid.*, 1953, 172, 76.

2. Alexander, P., Charlesby, A., and Ross, M., *Proc. Roy. Soc. London*, 1954, A223, 392. Alexander, P., Charlesby, A., and Black, R. M., *ibid.*, 1955, A232, 31. Dole, M., Keeling, C. D., and Rosa, D. G., *J. Am. Chem. Soc.*, 76, 4304 (1954).

3 Alexander, P., and Fox, M., *Nature*, 1952, 169, 572; *Trans. Faraday Soc.*, 1954, 50, 605.

4 Alexander, P., and Charlesby, A., *Nature*, 1954, 173, 578. Alexander, P., Charlesby, A., and Ross, M., *Proc. Roy. Soc. London*, 1954, A223, 392. Alexander, P., and Charlesby, A., *ibid.*, 1955, A230, 136.

5. See, for example, Bacq, S. M., and Alexander, P., *Fundamentals of Radiobiology*, Butterworth, London, 1955

6. Stacey, K. A., and Alexander, P., International Symposium on Macromolecular Chemistry, Milan-Torino, 1954. Published in *Ricercia sci.*, 25, 889 (1955). Distributed in the U. S. by Interscience, N. Y.

7 Alexander, P., and Hitch, S F., *Arch. Biochem & Biophys.*, 1952, 9, 229.

8. Alexander P , and Stacey, K. A., *Trans. Faraday Soc.*, 1955, 51, 299.

9. Alexander, P., and Fox, M., *J. chim. phys.*, 1953, 50, 415.

10. Magat, M., and Wall, L. A., *ibid.*, 1953, 50, 308.

11. Alexander, P., and Fox, M., *ibid.*, 1955, 52, 711.

12. Charlesby, A., *Proc. Roy. Soc. London*, 1954, A222, 542.

13. Charlesby, A., and Alexander, P., *J. chim. phys.*, 1955, 52, 718.

14. Charlesby, A., *Proc. Roy. Soc. London*, 1955, A231, 521.

$$r_{gel} = \frac{Ne}{aM_n} \frac{ac}{4 - 5 \exp\{-ac\}}$$

where N is Avogadro's number, e is the energy absorbed per activated mono-
mer unit, and M_n is the initial number-average molecular weight. The
relationship between r_g and the concentration c is shown in Figure 7. No
gel formation occurs at concentrations such that $ac < 0.223$ and the mini-
mum radiation dose for gel formation occurs at concentration some three
times greater when $r_{gel} = 0.46 \, Ne/aM_n$. The upward sweep of the gelling
dose at higher concentrations arises from the absence of degradation at these
concentrations and the increasingly higher number of polymer molecules
available to absorb the same amount of radiation energy. Such an upward
sweep is in fact observed, although it is not always so steep. A lower slope
is obtained if less energy is required to activate a monomer unit by direct
rather than by indirect action.

The only parameters in the theory are a and e. For many polymers, gel
formation only occurs at concentrations exceeding 0.4% so that $ac = 0.223$
at $c = 4 \times 10^{-3}$ and a is about 50. The minimum gelling dose is found at
concentrations of about 1–2%; the theory predicts a minimum at $ac \sim$
0.8 so that once again $a \sim 50$. Theory gives a minimum value for the gelling
dose of:

$$(0.46Ne/aM_n) \text{ e.v./g.}$$

about $9 \times 10^7 \, e/M_n$ roentgens. For polyacrylic acid, the minimum gelling
doses lead to a value for e of 100 e.v. This compares well with the value
for direct action, deduced above for dry polymer of 104 e.v.

Figure 8 shows the experimental gelling doses for polyvinylpyrrolidone,
assuming $a = 55$. Good agreement with the theoretical curve is obtained
if $M_n/e \sim 10^3$ (which is the correct order of magnitude since, if $e \sim 100$ e.v.,
$M_n \sim 10^5$). In particular, the theoretical curve reproduces the sharp mini-
mum and subsequent slow rise with increasing concentration. If energy
absorbed directly on the polymer molecule is more effective than the in-
direct effect, the general shape of the curve is almost unaffected but the slope
at higher concentrations is reduced.

(5) **Conclusions.** Apart from mechanism (1) there are no decisive
experiments to distinguish between the other three mechanisms described.
The fact that polymers which crosslink at higher concentrations degrade
at very low concentrations (see, for example, polyacrylic acid and poly-
acrylamide, Tables III and IV) is not decisive evidence against mechanism
(2) since the existence of an oxygen effect complicates the situation.
One would expect that the influence of oxygen would be most marked in
low polymer concentrations where it might be responsible for converting a
labile point into a main chain break. At higher concentrations interaction
with another molecule would be more likely so that the chance for oxygen
to intervene might be less. The basic problem which remains to be solved
is what are the chemical reactions which bring about the crosslinking?

In spite of widely differing constitution the active agents all share the property of reacting under physiological conditions with nucleophilic (i.e. electron-rich) centres; and that their biological activity is derived from the ability to change some vital molecules in the cell by alkylation seems certain.

There are very many points at which these agents can react in the cell, and the most important are amino (or imino) groups in their uncharged state and anions such as the ionized carboxyl groups in proteins and the phosphate groups of nucleic acid. Ionized —SH groups (i.e. —S⁻) react so readily with these alkylating agents that even at pH 7 all the available —SH groups are readily alkylated although less than $0 \cdot 1$ per cent of them are present in the reactive —S⁻ form at any one time.

In spite of the great diversity of possible reactions it is possible to eliminate a number of these by considering compounds which are inactive although they have some of these reactions in common. Hendry, Rose and Walpole (1951) examined a large number of compounds which were known to react with amino groups by alkylation (such as the alkyl halides), by acylation or in other ways, but which could not esterify anions. Although they were not tested by these authors for mutagenic properties none showed cytotoxic effects on tumours which are such a characteristic feature of the radiomimetic alkylating agents. Similar negative results have been obtained more recently by Haddow (unpublished) with a number of bifunctional thiol esters of the type $R'S \cdot CORCO \cdot S \cdot R'$ which combine with amine groups under biological conditions. We studied in some detail one of the amine reactors used by Hendry, Rose and Walpole (1951), triacrylformal,

$$
\begin{array}{c}
\text{N—CO} \cdot \text{CH}{=}\text{CH}_2 \\
\diagup \diagdown \\
\text{CH}_2 \ \ \text{CH}_2 \\
| \quad \quad | \\
\text{CH}_2{=}\text{CH} \cdot \text{CO} \cdot \text{N} \ \ \text{N} \cdot \text{CO} \cdot \text{CH}{=}\text{CH}_2 \\
\diagdown \diagup \\
\text{CH}_2
\end{array}
$$

THE REACTIONS OF THE MUTAGENIC ALKYLATING AGENTS WITH PROTEINS AND NUCLEIC ACIDS

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A WIDE variety of different substances are now known to be mutagenic but the most active are the nucleophilic alkylating agents of which mustard gas—the first substance to be recognized as mutagenic (Auerbach and Robson, 1944)—is a typical representative. Alkylating groups which confer biological activity are (cf. Fahmy and Fahmy, 1956):

$-S \cdot CH_2 \cdot CH_2 \cdot Cl$	Sulphur mustard
$-N \cdot CH_2 \cdot CH_2Cl$	nitrogen mustard
$R \cdot O \cdot SO_2 \cdot CH_3$	mesyloxy
$R \cdot CH\!\!-\!\!CH_2$ $\diagdown O \diagup$	epoxide
$CH_2\!\!-\!\!CH_2$ $\diagdown N \diagup$ $\mid$ R	ethylene imine
$R \cdot CH\!\!-\!\!CH_2$ $\mid \quad \mid$ $O\!\!-\!\!CO$	propiolactone

They are often referred to as radiomimetic alkylating agents (Dustin, 1947) since the biological effects they produce at the cellular level simulate closely those which follow exposure to ionizing radiations.

Sir Rudolph Peters (1947) first suggested that the ability to esterify acid groups *in vivo* might be responsible for the biological activity of mustard gas. In the subsequent ten years this suggestion has received a large measure of support.

every case the agent was present in great excess. Very little
is known of the combination of the other types of radiomimetic
alkylating agents with proteins and this led us to examine
systematically the reaction of a representative number of
these with serum albumin under physiological conditions.
The reaction with the different groups in the intact proteins
was determined by a variety of methods (Cousens, 1956). The
results, summarized in Table I, indicate that the most pro-
minent reaction common to all the biologically active alkylat-

Table I

CHANGES PRODUCED IN SERUM ALBUMIN BY RADIOMIMETIC
AGENTS

Treatment	% Reduction			
	Carboxyl Groups	Amino Groups	Imidazole Groups	Tyrosine
O=[CH₂·CH—CH₂]₂ \O/	27	46	77	No reaction
CH₂·CH—CH₂ \O/	22	60	70	No reaction
Myleran	31	0	42	No reaction
Chlorambucil	25	7	5	No reaction

3% Protein Solution + 0 12 Moles of Reagent

ing agents is esterification of the carboxyl groups. The
epoxides reacted extensively with amino groups of lysine
and the imino group of histidine; the nitrogen mustards
reacted to a lesser extent and the mesyloxy compounds hardly
at all.

Chromatographic examination of the acid hydrolysate of
the reacted proteins showed an almost complete disappear-
ance of the spot due to histidine after reaction with epoxides
and a very noticeable decrease after treatment with nitrogen
mustard. The intensity of the lysine spot was also changed.
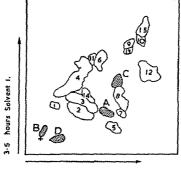
and found that it very readily alkylated amino groups in pro-
teins, but Fahmy and Fahmy (unpublished) were not able to
produce any mutations in *Drosophila* with it. Similarly
specific thiol reagents which inactivate sulphydryl enzymes
in the cell (cf. Ross, 1953) do not bring about the highly
specific forms of genetic damage associated with radiation
and the alkylating agents belonging to the mustard class.
These experiments indicate that reaction *in vivo* with amino
or sulphydryl groups does not produce radiomimetic effects
and the biologically significant reaction of the alkylating
agents considered in this paper would appear to be the
esterification of acid groups. This type of reaction can only
be brought about under physiological conditions by sub-
stances most of which are mutagenic, and cannot be pro-
duced with the inactive substances.

The *in vitro* reaction with nucleic acids and proteins lends
powerful support to the arguments just advanced that
combination with acid groups is the biologically important
reaction. That is not to say that this is the only or even
the predominant reaction which takes place with cellular
constituents, and a substantial proportion of the reagents are
probably wasted in useless reactions. The important process
can therefore not be discovered merely by following the *in vivo*
fate of an active compound. The experiments described in
this paper were designed to throw light on the mechanism by
which these reagents exert their biological activity, although
general considerations strongly suggest that the relevant
reactions of chemical mutagens must be with the nucleic acid
moiety of nucleoprotein. A summary of our results with
proteins is given for the sake of completeness.

Reaction with Proteins

A number of papers have been published on the reaction
of proteins with mustard gas (see review by Alexander, 1954)
but the results do not provide information about the relative
reactivities of different groups in the proteins since in almost

3·5 hours Solvent 2.

Fig. 1. Two-dimensional chromatogram of hydrolysate of serum albumin. Solvent I contains ethanol, butanol, water and propionic acid. Solvent II contains acetone, butanol, water and dicyclohexylamine. The following new spots were seen after treatment with these different alkylating agents:

A with (CH₃)₂.N.CH₂.CH₂Cl
B with CH₃.N:(CH₂.CH₂.Cl)₂

C with CH₃.CH . CH₂
              \O/

D with (CH₂—CH.CH₂)₂O
              \O/

Key to the other spots:

1	Arginine	9	{ Valine / Methionine
2	Lysine		
3	Aspartic acid	10	Phenylalanine·
4	Glutamic acid	11	Proline
5	Cystine	12	Threonine
6	Alanine	13	Tyrosine
7	Histidine	14	Glycine
8	Serine	15	{ Leucine / Isoleucine

No alteration could be seen in the chromatogram of the mesyloxy-treated proteins; this is to be expected since the esters formed with the carboxyl groups would be broken down again under the conditions used to hydrolyse the protein into its constituent amino acids.

The epoxide- and nitrogen mustard-treated proteins gave rise to additional spots on the chromatogram which are probably due to N-alkylated lysine or histidine (Fig. 1). The new spots from proteins treated with bifunctional·reagent are close to the origin and this would be consistent with the cross-linking of two amino acids to give a large molecule which diffuses slowly.

The reaction with protein SH groups was not studied in serum albumin since this contains less than one SH group per molecule and ovalbumin was used instead. In the native protein no significant reaction could be obtained (Table II) but

Table II

REACTION OF ALKYLATING AGENTS WITH THE —SH GROUP

3% egg albumin before and after denaturation

Alkylating agent*	% SH groups reacted	
	Native protein	Denatured protein†
(ClCH$_2$·CH$_2$)$_2$N·C$_6$H$_4$·(CH$_2$)$_3$ COOH Chlorambucil (1·7% solution)	14	88
CH$_3$·SO$_2$·O·C$_2$H$_5$ (2·6% solution)	0	55
CH$_2$—CH CH$_3$ (5% solution) \O/	0	100

* Reaction time 4 hours at 37° C.   † Denatured by guanidine.

this was to be expected since these groups had earlier been found inaccessible to thiol reagents. After denaturation with guanidine, the —SH groups reacted extremely readily with the three reagents tested. With mustard gas, Bacq (1946) showed that the rate of combination increased rapidly as the pH was raised from 6 to 9 and this is in agreement with the theoretical prediction that the —S⁻ form is the reactive species.

But on esterification no acid is released:

$$>NCH_2 \cdot CH_2 \cdot Cl + = \overset{|}{P}O^- Na^+ \longrightarrow >NCH_2 \cdot CH_2 \cdot O\overset{|}{P} = + NaCl.$$

With relatively dilute solutions of DNA a considerable amount of acid is produced during the reaction with the
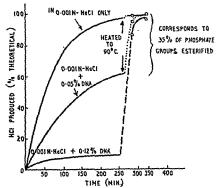
FIG. 2. Rate of acid formation when the phenylalanine nitrogen

mustard. That the liberation of this acid is entirely due to hydrolysis by water and not produced in part by alkylation of amino groups was shown by analysis of the solution after reaction was complete. The amount of mustard which had combined with the DNA was equal to that bound by esterification as calculated from the decrease in the amount of acid released, while the amount of hydrolysed mustard found

Reaction with nucleic acids
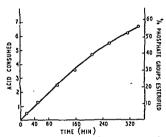
Our attention has so far been confined almost entirely to DNA*; in this substance one might expect reaction with the phosphate and the amino imino groups and ring nitrogen of the purine and pyrimidines, all of which are in the electrophilic (or reactive) form at pH 7. With mustard gas—present in very large excess—Elmore and co-workers (1948) obtained extensive esterification of the phosphate groups of DNA and indication for reactions with amino groups based on changes in the titration curve, but more recent work on the titration behaviour of DNA invalidates their interpretation. Alexander (1952) found that all the other types of radiomimetic alkylating agents readily esterified the phosphate groups of DNA under mild conditions.

With the biologically highly active mustard from l-phenylalanine (Bergel and Stock, 1954), i.e. CB 3025.

$$COOH \cdot CH \cdot CH_2 \underset{\underset{NH_2}{|}}{\bigcirc} N \underset{CH_2 \cdot CH_2 \cdot Cl}{\overset{CH_2 \cdot CH_2 \cdot Cl}{<}}$$

we were able to show, when the mustard was not present in excess, that the only reaction which could be detected with native DNA is esterification of the phosphate groups. By using an automatic titrator the rate of release of acid due to hydrolysis of the mustard group could be followed while maintaining the pH throughout at 7. In the presence of DNA the amount of acid produced is decreased (Fig. 2) and this provides a direct measure of the number of phosphate groups which have been esterified. Acid is liberated to the same extent when the mustard group is hydrolysed by the water or when it alkylates an amino group:

$$>NCH_2 \cdot CH_2 \cdot Cl + H_2O \longrightarrow >NCH_2 \cdot CH_2 \cdot OH + HCl$$
$$>NCH_2 \cdot CH_2 \cdot Cl + RNH_2 \longrightarrow >NCH_2 \cdot CH_2 \cdot NHR + HCl.$$

* The DNA we used was obtained from the sperm heads of herring by a modification of the method of N...

Since the epoxides react much more slowly than the mustards and also since the proportion which hydrolyses is much greater, it is not possible to determine the extent of combination with amino groups. Using excess epoxide the absorption spectrum of the DNA is slightly altered after 50 per cent of the phosphate groups have become esterified, and this indicates that some reaction with the purine or pyrimidine bases has occurred. No change in the spectrum was observed immediately after comparable treatment with aliphatic nitrogen

Fig. 3. Rate of alkali produced (i.e., acid consumed to maintain pH at 7) when 0·1% DNA was treated with a 2% solution of propylene oxide at 37° C.

mustards and this is another illustration of the greater reactivity of epoxides with amino groups (cf. Table I).

On extending our investigations to other aromatic mustards we found that some did not combine significantly with DNA in solution. A notable example was chlorambucil,

$$COOH(CH_2)_3 - \bigcirc - N \begin{cases} CH_2 \cdot CH_2 \cdot Cl \\ CH_2 \cdot CH_2 \cdot Cl \end{cases}$$

a compound used clinically and found by Fahmy and Fahmy (1956) to have the highest mutagenic activity of any mustard.

free in the solution after reaction corresponded exactly to the amount of acid produced.

The absence of reaction with amino groups is shown still more strikingly with solutions containing 0·12 per cent DNA when 94 per cent of the mustard is used up in esterification (Fig. 2) and only 6 per cent of it liberates acid. This 6 per cent is entirely due to hydrolysis.

In agreement with the prediction of Brown and Todd (1952) we find that the triesters produced by alkylation of the phosphate groups of DNA are unstable and are hydrolysed completely by heating at 90° C for 20 minutes. Fig. 2 shows that after this treatment the amount of acidity produced is equal to the amount of esterification (i.e. after boiling, the total amount of acid produced is equal to that which would have been produced by hydrolysis in the absence of DNA). Within experimental error (± 5 per cent) all the combined mustard residues were released on hydrolysis and this shows that the triester breaks down predominantly at the mustard linkage. Measure-

$$
\underset{\overset{\mid}{O}}{\overset{\overset{\mid}{O}}{O}} = P\text{---}OCH_2\cdot CH_2\cdot N\langle \xrightarrow{H_2O} \underset{\overset{\mid}{O}}{\overset{\overset{\mid}{O}}{O}} = P\text{---}O^- + OHCH_2\cdot CH_2\cdot N\langle
$$

ment of the molecular weight shows that breakdown also occurs, though very infrequently, at one of the sugar esters. When this happens a break in the main chain is produced (see p. 314).

With epoxides, esterification of the mustard groups is also the most prominent reaction. In this case it results in the liberation of alkali which was followed with the automatic titrator (Fig. 3).

$$
\underset{\overset{\diagdown}{O}\diagup}{CH_2\text{---}CH\text{---}R} + \, = \underset{\overset{\mid}{\vert}}{\overset{\overset{\mid}{\vert}}{P}}\text{---}O^-Na^+ \longrightarrow
$$

$$
= \underset{\overset{\mid}{\vert}}{\overset{\overset{\mid}{\vert}}{P}}\text{---}O\cdot CH_2\cdot \underset{\overset{\mid}{OH}}{CH}\text{---}R + NaOH.
$$

with a positively and a negatively charged synthetic polymer (polyethylene imine and polymethacrylic acid respectively) show that reaction is very largely influenced by electrostatic repulsion or attraction between the reactants.

Reaction with nucleoproteins

In the cell, of course, DNA is not present as such but combined with proteins which will reduce its electrical charge. Consequently we decided to study the reaction of a nucleoprotein with the radiomimetic alkylating agent. Since the extraction of nuclear proteins from the cell gives ill defined products which are difficult to handle, we used sperm heads obtained by plasmolysing soft roe from herrings (Felix, Fischer and Krekels, 1956). These are hard balls with a diameter of the order of 2 μ, containing only DNA and the low-molecular weight proteins, the protamines, in the ratio of 39 per cent to 61 per cent. They are surrounded by a tough membrane the weight of which is insignificant. When placed in water they swell only to a very limited extent and take up less than their own weight of water. Suspensions of sperm heads can therefore be handled easily and they are not at all viscous.

The reaction of the sperm heads with the alkylating agents can be followed on the automatic titrator in exactly the same way as with the solution of DNA. From the amount of acid liberated the esterification of the phosphate groups can be determined and is seen to occur extremely readily (Fig. 4). For the aromatic mustards it could again be shown that the only reaction which occurred was esterification of acid groups and there was no evidence of alkylation of amino or imino groups in either the protein or the nucleic acid. Since the number of carboxyl groups in the protein is very much less than that of the phosphate groups from DNA the esterification of the latter would again appear to be the predominant reaction. Other evidence (see p. 311) supports the view that the reaction of the mustard is confined to DNA and that the protamine is not involved.

These observations seemed to be very difficult to reconcile
with the view that the radiomimetic alkylating agents exerted
their effect by combining with DNA, and led us to examine
the reaction of a series of water-soluble aromatic nitrogen
mustards with proteins and DNA. The relative rates of reac-
tion were expressed as a competition factor (Ogston, 1948)
which was defined as:

$$\frac{\text{Amount of mustard combined with macromolecule}}{\text{Amount of mustard hydrolysed by water}} \times \frac{1}{\text{concentration of macromolecule}}$$

The concentration of macromolecule was expressed as a
molarity based on the total number of groups present which
can be alkylated. Table III shows that there is no correlation

Table III

COMPETITION FACTORS OF AROMATIC NITROGEN MUSTARDS FOR
PROTEINS : DNA

Compound (C CH$_2$CH$_2$)$_2$N—⟨⟩—R	Biological Activity	Serum Albumin	Serum Globulin	Fibrin- ogen	DNA	Poly- metha- crylate	Poly- ethylene imine
R							
CH$_2$·CH (NH$_2$) COOH (Phenylalanine Mustard)	+ + +	26	23	—	180	2100	21
O (CH$_2$)$_2$N·(CH$_3$)$_2$Br⁻	—	40	7	2	460	1400	20
(CH$_2$)$_2$N·(CH$_3$)$_2$	+ +	20	—	—	310	4400	—
(CH$_2$)$_2$·NH$_2$	+ +	25	—	—	190	2000	—
—COOH	+	31	20	5	0	0	240
(CH$_2$)$_3$·COOH (Chlorambucil)	+ + +	74	20	20	20	100	170

between reactivity with DNA and biological activity. All
mustards carrying a positive charge reacted readily while
those like chlorambucil which have a negative charge (i.e. the
ionized carboxyl group) at pH 7 fail to react. Experiments

The negatively charged mustard, chlorambucil, reacts readily with the sperm heads (Fig. 5) presumably because unlike the nucleoproteins it does not carry a negative charge which repels the mustard. The apparent paradox of a highly mutagenic mustard which does not react with DNA has thus been eliminated.

Reaction with RNA

RNA obtained by the method of Kay, Simmons and Dounce (1953) from rat liver was reacted with the phenyl-alanine mustard. The course of the reaction was followed on
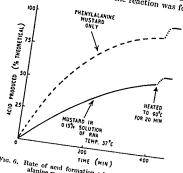
FIG. 6. Rate of acid formation when 0·1% phenyl-alanine mustard reacts with RNA.

the automatic titrator (Fig. 6) and the reduction in acid production shows that as with DNA there is very extensive reaction with phosphate groups. But the esters formed are stable and no appreciable acidity was produced on heating. Since reaction with the primary phosphate groups would give
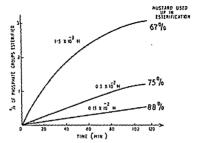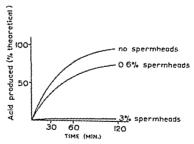
FIG. 4. Reaction of three different concentrations of phenylalanine mustard with a 10% suspension of herring sperm heads. The percentage of esterification was computed from the amount of acid liberated.

FIG. 5. Reaction of herring sperm heads with ... ...

protein occurs.

When the reaction occurs within the same molecule it causes a reduction in viscosity (Alexander and Fox, 1952) because the molecule is coiled up so that it occupies less volume*. On intermolecular reaction the viscosity of the solution rises steadily until quite suddenly it sets to a gel, when the number of crosslinks formed exceeds, on average, one per molecule.
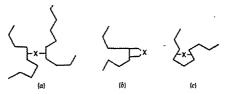
Fig. 7. Different types of reactions produced by bifunctional reagents with a flexible macromolecule like DNA

(a) Intermolecular crosslinking.
(b) Reaction with neighbouring groups.
(c) Intramolecular crosslinking.

Internal crosslinking can be recognized very clearly from light scattering measurements. By plotting the light scattered at different angles, as shown in Fig. 8, the volume occupied by the molecule in solution (expressed as the radius of gyration) is given by the slope and the molecular weight by the intercept (the smaller the value for the intercept the higher the molecular weight). Using this technique it is clear that the first change produced by a polyfunctional reagent is to

triesters which should be less stable than those from DNA (Brown and Todd, 1952) it seems highly improbable that the reduction in the amount of acid produced is due to esterification of the primary phosphate groups. The stable esters which are formed may be diesters derived from esterification of the secondary phosphate groups at the end of the chain. These, of course, would be very much more numerous in the relatively low-molecular weight RNA than in DNA. However, this whole reaction needs much more detailed study.

Crosslinking of DNA

When assessed by growth inhibition and the production of chromosome breaks, the biological activity of alkylating agents containing more than one reactive group per molecule is in general much greater than that of monofunctional compounds (Haddow, 1953). Although a direct comparison has only been made in relatively few cases (Fahmy and Fahmy, 1956; Zamenhof et al., 1956) this same difference seems to persist also for mutagenicity.

In principle, there are three reactions which distinguish a bifunctional from a monofunctional reagent: the ability to form crosslinks between different molecules (intermolecular reaction); to join different groups in the same molecule (intramolecular reaction) and to form rings by reacting twice with the same group (e.g. $RNH_2 + X \cdot M \cdot X \rightarrow RN)M + 2HX$) or on two adjacent groups (Fig. 7). Since compounds which are sterically prevented from forming a ring by double alkylation such as $CH_3 \cdot SO_2O \cdot CH_2 \cdot C \equiv C \cdot CH_2 \cdot O \cdot SO_2CH_3$ are highly mutagenic. this last possibility need not be considered.

With a relatively flexible macromolecule like DNA, intramolecular crosslinking will predominate in dilute solution, while in more concentrated solutions reaction between molecules will take place. Using synthetic polymers (Stacey et al., 1957) the change-over from one type of mechanism to the other was followed in detail and was found to take place over a remarkably small range of concentration.
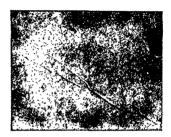
Fig. 9a. An electron micrograph of DNA which shows the tendency of two molecules to come together as a side-by-side aggregate under the conditions used in these experiments.

Fig. 9b. An electron micrograph at a lower magnification which shows how, under these conditions, native DNA is stretched out by surface tension.
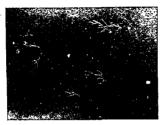
FIG. 9c. An electron micrograph of DNA after
reaction with phenylalanine mustard. The cross-
links have prevented the straightening and the
enforced proximity of different points of the chain
has facilitated the side-by-side aggregation and
has caused the collapse of the molecule into
these shapes.

Table IV

THE INITIAL EFFECT OF ALKYLATION ON THE SIZE OF DNA

Reagent	%	Molecular weight	Radius of gyration
Control	—	6.4×10^6	2000
CB. 3025 (phenyl-	6	6.0×10^6	1100
alanine mustard)	~20	5.4×10^6	980
	21	6.0×10^6	1080
	26	2.7×10^6	350
	34	8.0×10^5	220
	53	8.0×10^5	200
HN2	~10	6×10^6	1530
	~25	4.8×10^6	1040
CB. 1348 (chlorambucil)	0	5.8×10^6	1800
Propylene oxide	49	6.7×10^6	2200
Bisepoxypropyl ether	20	20×10^6	1350
	42	7.4×10^6	875

effect of internal crosslinking, and the difference in appearance between untreated * DNA and DNA after reaction with a nitrogen mustard is most marked. On drying, the various strands in the internally crosslinked DNA molecule are pulled together to give the patterns seen. In solution, the electrostatic repulsion of the phosphate groups opens up the molecule into a more sponge-like structure.

Intermolecular crosslinking of DNA is found after treatment of the sperm heads with bifunctional alkylating agents. The untreated nucleoprotamine dissolves completely in 2 M sodium chloride to give highly viscous solutions but after reaction with bifunctional alkylating agents a proportion of the nucleoprotamine is present as a highly swollen gel, the presence of which cannot usually be detected by inspection. Centrifuging for one hour at 20,000 g spins down this gel without producing any sediment in the control solution. The proportion of DNA present as gel after a variety of treatments is shown in Table V. The gel is composed entirely of DNA and contains no protamine. This means that the crosslinking

---

* DNA molecules are not rigid rods and the linear appearance of the untreated DNA is brought about by surface tension during drying which pulls out the molecules.

coil up the molecule (see Table IV) and only after very extensive reaction is there a fall in molecular weight (this will be discussed on p. 315). With a monofunctional reagent such
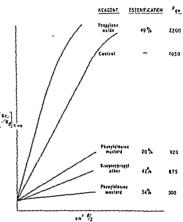
FIG. 8. Light scattering plots (the reciprocal of the scattering at each angle is plotted as a function of that angle), for DNA treated with various alkylating agents.

$\rho g.w$, the radius of gyration which is obtained from the slope of the corresponding line, is a measurement of the extension in space of the DNA molecule after reaction. The molecular weight is inversely proportional to the intercept.

as propylene oxide no change in shape or molecular weight is observed, proving that esterification of the phosphate groups alone is not sufficient to coil up the molecule.

Electron microscope photographs (Fig. 9) also reveal the

Many radiomimetic effects such as growth inhibition can probably be explained on a DNA "deletion" hypothesis, i.e. they are the result of rendering DNA, in one way or another, useless to the cell. Obviously, crosslinking both intra- and intermolecular will be most effective for this purpose since a very few reactions per molecule entirely alter its physical character and are bound to render it biologically inactive. Monofunctional reagents, on the other hand, will only esterify isolated phosphate groups and it seems very reasonable that the blocking of a few of these may be sufficient to spoil the molecule and that a number of these random esterifications are necessary before biological activity is lost. This would explain why higher concentrations of the monofunctional compounds have to be used to produce the same effect.

Probably the lethal as well as some *visible and biochemical* mutations can be satisfactorily explained by this DNA "deletion" hypothesis since they are manifestations of the loss of a gene. The major difficulty is to explain how non-selective reactions like the alkylation of phosphate groups can produce a change in gene function as opposed to a general destruction of the molecule for biological purposes. We must look to much more subtle processes than crosslinking for causing a rearrangement of a DNA molecule so that it retains its biological integrity but carries a different code. Although we are in no position to put forward any hypothesis, experiments on the production of isolated breaks in one of the chains of the twin molecules may provide the basis for a possible mechanism.

The possible significance of "hidden breaks"

A break produced in one of the twin chains of DNA does not lead to a disruption of the molecule since this is maintained intact by the other chain. A decrease in the particle size *will only occur when there are interruptions in both* chains close together (i.e. within about 50 A) (Fig. 10). The hidden breaks can, however, be revealed if the twin chains are

separated and this can be done with DNA from herring sperm (Alexander and Stacey, 1955a and b; 1957a), by exposing a dilute solution to 4M urea when the molecular weight is halved, from 6×10^6 to 3×10^6, without affecting the length of the molecule. After irradiation with X-rays the molecular weight of the DNA when measured in salt falls much less rapidly than the molecular weight determined after dissociation by urea, because eight out of ten of the breaks occur in

Fig. 10. A diagrammatic representation of the effect of urea on irradiated DNA which shows how breaks hidden in the dimeric structure (b) become apparent in urea solution when all the hydrogen bonds are broken.

one of the chains only and remain hidden until the molecule is split* (Alexander and Stacey, 1956). When α-rays are used no hidden breaks are produced because the destruction along the track of the particle is so intense that both chains are invariably broken when crossed by an α-particle (Stacey and Alexander, 1957).

Breaks in the chain are also produced by the alkylating

---

* The double breaks are not due to a chance event whereby two ionizations are produced close together in each of the two chains. They are probably produced by the densely ionizing tail portion of each electron track.

agents as a result of the instability of the triesters produced. Although in the majority of the hydrolyses the alkylating agent is split off again, occasionally the sugar ester is hydrolysed and this brings with it an interruption of the chain. Table VI illustrates the change (on standing at 37° C) in

Table VI

PRODUCTION OF "SINGLE" AND "DOUBLE" BREAKS IN DNA

Reagent used	% Esterification	Molecular weight × 10⁴	
		in salt soln.	in urea soln.
None	0	6–6·4	3–3·5
propylene oxide			
24 hrs. at 0° C	49	6·7	0·2
24 hrs. at 37° C		3·3	0·1
butadiene bisepoxide			
24 hrs. at 0° C	42	7·5	3·2
24 hrs. at 37° C		2·15	0·77
l-phenylalanine mustard			
24 hrs. at 0° C†	3	6·0	2·85
24 hrs. at 37° C		6·0	2·25
24 hrs. at 0° C	20	5·4	1·8
24 hrs. at 37° C		3·05	1·1
24 hrs. at 0° C	27	2·7	1·05
60 hrs. at 37° C		0·15	—
24 hrs. at 0° C	53	0·8	—

† Even this small degree of esterification was sufficient to bring about appreciable internal crosslinking.

molecular weight due to hydrolysis of DNA treated with alkylating agent. The existence of hidden breaks is very clearly revealed after extensive treatment with the monofunctional epoxide, propylene oxide. The molecular weight of the double molecule is unchanged while after splitting it is reduced to approximately one-fifteenth. With the bifunctional reagents the number of hidden breaks cannot be determined since the two chains are covalently crosslinked and this prevents the splitting into two strands by the urea except when there are breaks in the main chain to compensate for

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Breaks in the chain are also produced by the alkylating

* The double breaks are not due to a chance event whereby two ionizations are produced close together in each of the two chains. They are probably produced by the densely ionizing tail portion of each electron track.

formed within one molecule which then becomes distorted.
When the nucleoprotamine is treated, crosslinks are formed
between different DNA molecules which are thereby rendered
insoluble and form a gel when the nucleoprotein is dispersed
in strong salt. The greater effectiveness of the bifunctional
reagents is attributed to crosslinking which is a much more
efficient process of spoiling DNA than the isolated blocking
of phosphate groups which is the only reaction produced by
the monofunctional reagents.

The triesters formed when the phosphate groups of DNA
are alkylated, are unstable and occasionally hydrolyse to give
a break in one of the polynucleotide strands of DNA. These
breaks are masked by the other chain of the twin molecule
and can only be revealed by special techniques. The formation
of these hidden breaks may provide a mechanism for the
rearrangement within a DNA molecule without at the same
time so altering its configuration as to make it useless to the
cell. In this way DNA might be made to transmit an altered
code.

REFERENCES

ALEXANDER, P. (1952). *Nature, Lond.,* 169, 226.
ALEXANDER, P. (1954). *Advanc. Cancer Res.,* 2, 1.
ALEXANDER, P., and FOX, M. (1952). *Nature, Lond.,* 169, 572.
ALEXANDER, P., and STACEY, K. A. (1955a). *Biochem. J.,* 60, 194.
ALEXANDER, P., and STACEY, K. A. (1955b). *Nature, Lond.,* 176, 162.

entia, 13, 307.
Lond., 154, 87.

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the crosslinks. The situation here is too complex to be analysed by the methods available to us.

Molecules with hidden breaks appear to retain all their normal properties and it is conceivable that they continue to exercise their biological function but that they transmit a slightly altered code.

Summary

Chemical considerations suggest that the essential characteristic of the radiomimetic alkylating agents (i.e. sulphur and nitrogen mustards, epoxides, ethylene imines and esters of methanesulphonic acid) is their ability to esterify anions such as the carboxyl groups of proteins and the phosphate groups of DNA and RNA. These substances can also alkylate amino and sulphydryl groups. These reactions, although they undoubtedly occur in the cell, are unlikely to be of biological importance since many other substances which can react with amino and SH groups but not with anions are biologically inactive.

In proteins, the radiomimetic alkylating agents react with all the available groups such as NH_2, SH and COOH but in DNA only esterification of the phosphate groups is found unless the reagent is present in excess. The steric configuration of the DNA probably screens the purine and pyrimidine bases and this is confirmed by the failure of substances, which readily combine with amino groups in proteins, to react with DNA under physiological conditions. Probably the reason why the radiomimetic alkylating agents are such effective mutagens is because they can combine with the most vulnerable group in the DNA, the phosphate group.

Esterification of phosphate groups is also the most prominent reaction when a nucleoprotein (herring sperm heads) is treated with nitrogen mustards. The protamine does not block the phosphate groups in such a way as to prevent alkylation.

The polyfunctional alkylating agents, which are much more active, crosslink DNA. In dilute solution the crosslinks are

A COMPARISON OF THE REACTIVITY OF THE DISULPHIDE BOND IN WOOL AND PEPTIDES

By P. ALEXANDER* and M. FOX*

Summary

Oxidation of various cystine peptides by chlorine (pH 2 and 10) and potassium permanganate (pH 2 and 9.2) failed to reveal any effect of the position of cystine in the peptide chain on this reaction. The cystine of peptides also containing tyrosine is not completely oxidized by chlorine at either pH, nor is it preferentially attacked by acid permanganate, although non-tyrosine peptides show both these types of behaviour. The clear-cut division of the disulphide bonds in wool into 25 per cent. oxidizable by acid permanganate and hypochlorite, and 75 per cent. not thus oxidizable can therefore not be explained by the way in which they are incorporated into the peptide chain, though the proximity of a tyrosine residue affects the reactivity of cystine residues with oxidizing agents.

I. INTRODUCTION

Phillips and co-workers (cf. Phillips 1946) in their investigation on the reaction of wool with alkali found that the disulphide linkages appeared to be divided into two groups, one of which reacted more rapidly than the other. A similar behaviour was observed with other reagents such as sodium bisulphite and thioglycollic acid and Phillips *et al.* (1946) claimed to have found some evidence for a further subdivision in the two main groups. Although other workers (e.g. Schuringa 1950) failed to confirm the earlier results in detail, there can be little doubt that not all the disulphide bonds react at the same rate with these reagents. Phillips tentatively ascribed these differences to variations in the polar amino acid on either side of the cystine residues. The recent discovery made simultaneously by Horio and Kondo (1953) and Mercer (1953) that there is a morphological division along the length of the fibre to produce a bilateral segmentation into *ortho-* and *para-*parts, may provide a satisfactory explanation for the difference in reactivity. Fraser, Lindley, and Rogers (1954) and Mercer (1954) have already proved that substances which react with the disulphide bond attack the *ortho-*part preferentially. The *para-*part contains a higher proportion of cystine, and the increased amount of cross-linking so introduced renders this part less accessible.

Alexander, Hudson, and Fox (1950) found that whereas chlorine dissolved in water at a pH less than 7 could oxidize all the disulphide bonds of wool, solutions of hypochlorite at pH 10 as well as acidic solutions of

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BUTLER, J. A. V. (1950). *Nature, Lond.,* 166, 18.

CHANUTIN, A., and GJESSING, E. C. (1946). *Cancer Research,* 6, 599.

COUSENS, S. F. (1956). Reactions of some Chemical Carcinogens with Macromolecules. Ph.D. Thesis, University of London.

DUSTIN, P. (1947). *Nature, Lond.,* 159, 794.

ELMORE, D. T., GULLAND, J. M., JORDAN, D. O., and TAYLOR, H. F. W. (1948). *Biochem. J.,* 42, 308.

FAHMY, O. G., and FAHMY, M. J. (1956). *J. Genet.,* 54, 146.

FELIX, K., FISCHER, M., and KREKELS, A. (1956). *Progress in Biophysics,* 6, 1.

HADDOW, A. (1953). *In* Physiopathology of Cancer, p. 475. New York: Hoeber.

HENDRY, J. A., ROSE, F. L., and WALPOLE, A. L. (1951). *Brit. J. Pharmacol.,* 6, 201.

KAY, E. R., SIMMONS, N. S., and DOUNCE, A. L. (1952). *J. Amer. chem. Soc.,* 74, 1724.

KAY, E. R., SIMMONS, N. S., and DOUNCE, A. L. (1953). *J. Amer. chem. Soc.,* 75, 4041.

OGSTON, A. G. (1948). *Trans. Faraday Soc.,* 44, 45.

PETERS, R. A. (1947). *Nature Lond.,* 159, 149.

ROSS, W. C. J. (1953). *Advanc. Cancer Res.,* 1, 397.

SPARROW, A. M., and ROSENFELD, F. M. (1946). *Science,* 104, 245.

STACEY, K. A., and ALEXANDER, P. (1957). *Trans. Faraday Soc.,* 53, 251.

STACEY, K. A., COBB, M., COUSENS, S. F., and ALEXANDER, P. (1957). *Ann. N.Y. Acad. Sci.,* in press.

ZAMENHOF, S., LEIDY, G., HAHN, E., and ALEXANDER, H. E. (1956). *J. Bact.,* 72, 1.

In the present paper the authors report an investigation into the reactions of a number of different cystine peptides with oxidizing agents to determine whether variation in the side chain environment could influence the reactivity of the disulphide bond. Particular attention was paid to peptides containing tyrosine, since the tyrosine residues in wool show a behaviour towards oxidizing agents similar to that of cystine (Alexander and Gough 1951). Thus only 30 per cent. of the wool cystine is oxidized by hypochlorite although all reacts with chlorine at pH 2. This suggested the possibility that the varying reactivities found may be connected with the mode of linkage of these two amino acids.

II. Experimental

(a) Preparation of Peptides

The method developed by Bailey (1950) was used. The *N*-carboxy anhydride of an amino acid was reacted with equivalent amounts of an amino-acid ester and of a base (e.g. triethylamine) at ~10 to 0°C.

$$R.CH \sim CO + H_2N.CHR'.COOC_2H_5 \qquad R.CH.CO.NH.CHR'.COOC_2H_5$$
$$NH \sim CO + N(C_2H_5)_3 \quad \rightarrow \quad NH.CO.O.NH(C_2H_5)_3.$$

with $>O$ on the left structure.

The carbamate was then decomposed by warming to 30 to 40°C in vacuo or by the addition of acid. The volatile base and carbon dioxide were evolved, leaving the peptide ester or peptide ester hydrochloride.

$$R.CH.CO.NH.CHR'.COOC_2H_5 \qquad R.CH.CO.NH.CHR'COOC_2H_5$$
$$NH.CO.O.NH(C_2H_5)_3 \quad \xrightarrow{heat} \quad NH_2 + CO_2 + N(C_2H_5)_3.$$

Essentially the same procedure was followed for all the peptides. Table 1 gives the nitrogen analyses for peptides or for their ester hydrochlorides. For clarity the formulae given are those of the free peptide. As an example the preparation of di-DL-alanyl-L-cystinylbisglycine will be described.

One mole of glycine ethyl ester was reacted with 1 mole of triethylamine and 0.5 mole of anhydrocarboxy-L-cystine in dioxan for 10 min. The solvent was removed by heating to 0°C *in vacuo*. The substituted carbamate was then decomposed by heating *in vacuo* at 30 to 40°C for 10 min. The L-cystinylbisglycine ethyl ester was dissolved in chloroform-dioxan mixture and equivalent amounts of triethylamine and anhydro-carboxy-DL-alanine were added. The same procedure as just described then gave DL-bisalanyl-L-cystinylbisglycine ethyl ester. This was converted to the hydrochloride with ethereal hydrochloric acid and the product recrystallized from an ethanol-ether mixture. Dioxan-chloroform mixtures were used instead of chloroform in some instances to keep the volume of solution as low as possible.

potassium permanganate† could react with only 25 per cent. of the cystine (see Fig. 1). In contrast to the Phillips fractions these observations are not due to rate effects, cystine analyses being performed only when all the chlorine or hypochlorite had reacted. After reacting with 25 per cent. of the cystine, hypochlorite and permanganate were reduced by other groups in the fibre.

The cystine groups found with the oxidizing agents were not the same as those found by Phillips, so that when, for example, one of the Phillips' cystine fractions was destroyed by alkali, 25 per cent. of the
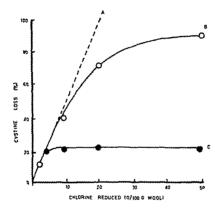
Fig. 1.—The oxidation of combined wool cystine with chlorine at pH 2 and hypochlorite at pH 10.

    A, Theoretical curve assuming oxidation to cysteic acid is the only reaction.
    B, At pH 2.
    C, At pH 10.

remaining cystine reacted with hypochlorite as in untreated wool. As the 25/75 fractionation was found also in hair and in wool whose structure had been disoriented by breaking hydrogen bonds in supercontraction, we felt (Alexander, Hudson, and Fox 1950) that morphological or steric factors in the crystalline region could not be responsible.

† Alkaline potassium permanganate only oxidizes the wool cystine after the wool has become degraded due to attack on other groups.

Bis-L-tyrosyl-L-cystine was prepared from the anhydrocarboxy-O-acetyl-L-tyrosine and L-cystine ethyl ester and the preparation carried out as described above as far as the peptide ester stage. This ester was then hydrolysed with an exact equiv. of $0.1N$ Ba(OH)$_2$ for 1 hr at room temperature followed by neutralization with $0.1N$ H$_2$SO$_4$, filtering to remove BaSO$_4$ and concentrating *in vacuo*. Under these conditions both the acetyl and ethyl groups were hydrolysed off, and the free peptide obtained. It was found that a better product was obtained when the peptide was taken to dryness on the steam-bath than when it was dried *in vacuo*.

(b) Oxidation of Peptides

The oxidizing agent, buffered where necessary, was added to 0.1 to 0.15 per cent. solution of the peptide in $0.05N$ HCl.

(i) *Chlorination at pH 2.*—The required amount of chlorine solution was measured with a graduated 1 ml pipette, and made up to 5 ml with water. This solution was added to 5 ml of the peptide solution in a conical flask. A drop of conc. H$_2$SO$_4$ was immediately added and the flask stoppered. 10 min was found to be sufficient time for the completion of the reaction. 5 ml of the solution was then used for analysis.

(ii) *Chlorination at pH 10.*—To the required volume of chlorine solution were added 1 ml of $1M$ KH$_2$PO$_4$ solution and a known vol. of $1N$ NaOH solution and the whole made up to 5 ml. The vol. of NaOH required was determined in trial experiments by measuring the pH of such solutions when added to 5 ml of peptide solution. The buffered chlorine solution was added to 5 ml peptide solution in a conical flask which was then stoppered and reaction was allowed to take place for 10 min. At the end of this time 5 ml of the solution was withdrawn for the colorimetric estimation of disulphide groups (see section IIc).

(iii) *Potassium Permanganate at pH 2.*—The required amount of a neutral permanganate solution of known strength was made up to 5 ml. This was added to 5 ml peptide solution in a conical flask together with 1 drop of conc. H$_2$SO$_4$. The flask was stoppered and reaction allowed to proceed for 15 min. At the end of this time, which was shown to be sufficient, 5 ml of the colourless solution were withdrawn for the estimation of disulphide.

(iv) *Potassium Permanganate at pH 9.2.*—The required amount of a neutral permanganate solution of known strength was made up to 5 ml with $0.1M$ borax solution. This was added to 5 ml peptide solution in a flask. The flask was stoppered and reaction proceeded for 15 min when it was shown that reaction was complete. The MnO$_2$ which had formed was filtered off and the solution analysed.

(c) Estimation of Disulphide Content

The colorimetric method of Shinohara (1935) using phospho-18-tungstic acid was employed since it had given very satisfactory results in

TABLE 1

ANALYSES OF PEPTIDES AND PEPTIDE ESTERS

Peptide	Formulae of Free Peptides	Total N_2* (%)	Amino N_2* van Slyke (%)
1. Bisglycyl-l-cystine methyl ester hydrochloride	$\left[H_2N.CH_2.CO.NH.\overset{\displaystyle COOH}{CH}.CH_2.S\text{—} \right]_2$	12.26 (12.30)	6.20 (6.15)
2. L-Cystinylbisglycine ethyl ester hydrochloride	$\left[HOOC.CH_2.NH.CO.\overset{\displaystyle NH_2}{CH}.CH_2.S\text{—} \right]_2$	11.50 (11.60)	5.73 (5.80)
3. Bis-DL-alanyl-l-cystinylbisglycine	$\left[H_2N.CH(CH_3).CO.NH.\overset{\displaystyle CH_2.S\text{—}}{CH}.CO.NH.CH_2.COOH \right]_2$	13.33 (13.41)	4.44 (4.48)
4. L-Cystinylbistyrosine ethyl ester hydrochloride	$\left[HO\text{—}C_6H_4\text{—}CH_2.\overset{\displaystyle COOH}{CH}.NH.CO.\overset{\displaystyle NH_2}{CH}.CH_2.S\text{—} \right]_2$	8.18 (8.06)	3.89 (4.03)
5. Bis-l-tyrosyl-l-cystine	$\left[HO\text{—}C_6H_4\text{—}CH_2.\overset{\displaystyle NH_2}{CH}.CO.NH.\overset{\displaystyle COOH}{CH}.CH_2.S\text{—} \right]_2$	9.89 (9.90)	4.87 (4.95)

* Theoretical values in brackets.

(2) L-Cystinylbisglycine:

$$\left[\begin{array}{c} NH_2 \\ | \\ HOOC.CH_2.NH.CO.CH.CH_2-S- \end{array} \right]_2$$

(3) Bis-DL-alanyl-L-cystinylbisglycine:

$$\left[\begin{array}{c} H_2N.CH.(CH_3).CO.NH.CH.CO.NH.CH_2.COOH \\ | \\ CH_2.S- \end{array} \right]_2$$

As glutathione

(4) L-Glutamyl-L-cysteinylglycine:

$$\left[\begin{array}{c} H_2N.CH.CH_2.CH_2.CO.NH.CH.CO.NH.CH_2.COOH, \\ | \qquad\qquad\qquad | \\ COOH \qquad\qquad CH_2.SH \end{array} \right]$$

was available, its reaction with chlorine and sodium hypochlorite was also examined.

In view of the similarity in behaviour of tyrosine and cystine, the reaction of

(5) Cystinylbis-L-tyrosine:

$$\left[\begin{array}{c} COOH \qquad NH_2 \\ | \qquad\qquad | \\ HO-C_6H_4-CH.CH.NH.CO.CH.CHS- \end{array} \right]_2$$

and

(6) Bis-L-tyrosyl-L-cystine:

$$\left[\begin{array}{c} NH_2 \qquad\qquad COOH \\ | \qquad\qquad\qquad | \\ HO-C_6H_4-CH_2.CH.CO.NH.CH.CH_2.S- \end{array} \right]_2$$

with oxidizing agents, was also examined.

(a) *Reaction with Chlorine and Hypochlorite*

If the mode of linkage affects the cystine reactivity, then it should prove possible to find a certain group of similarly constituted cystine peptides in which the disulphide bond reacts equally with chlorine at pH 2 and sodium hypochlorite at pH 10, and another group of cystine peptides in which there is a marked difference in the reactivity of the disulphide bond with these two reagents.

Two sets of experiments were devised to test this hypothesis. In the first, the total quantity of chlorine and hypochlorite respectively, required to oxidize all the combined cystine, was determined. In the second, the rate of loss of cystine and chlorine for oxidations at pH 10 was compared for the three peptides since a difference in the rate of reaction at pH 2 and 10 may in fact appear as a qualitative difference in the mixture of different peptides which make up a protein.

the estimation of cystine in protein hydrolysates. The reduction of the disulphide bond is carried out with NaHSO, and complete reduction is observed at pH 4.8 with cystine. For some of the peptides however, more alkaline solutions had to be used for maximum colour development. The pentapeptide bisalanylcystinylbisglycine reacted fully with phosphotungstic acid only at pH 5.7. The two tyrosine-cystine peptides, tyrosylcystine and bis(tyrosylalanyl)cystine however, required a still higher pH value for full colour development. The value of 86 per cent. for the tyrosylcystine attained at pH 7 was probably a true maximum since it agreed with the cystine analysis after complete hydrolysis of the peptide. The very high colour value obtained with cystinyl-bis(alanyltyrosine) cannot be explained

TABLE 2

MAXIMUM COLOUR INTENSITIES OF SOME CYSTINE PEPTIDES OBTAINED WITH THE PHOSPHOTUNGSTIC ACID REAGENT, WHEN REDUCED WITH BISULPHITE AT VARIOUS pH VALUES

Peptide	Colour Val. (% of calculated)			After* Hydrolysis to Cystine
	pH 4.8	pH 5.7	pH 7.0	
L-Cystinylbisglycine	97.1	97.1	—	—
Bisglycyl-L-cystine	106.0	106.0	—	—
Bis-DL-alanyl-L-cystinylbisglycine	81.4	100.5	—	—
Bis-L-tyrosyl-L-cystine	—	63.7	86.0	85.0
L-Cystinylbis-L-tyrosine	—	106.5	109.0	102.0
L-Cystinylbis-(DL-alanyl-L-tyrosine)	—	152.5	161.8	109.0
Glutathione	—	—	84.2	—

* Hydrolysed with 16% HCl for 16 hr at 125°C.

since analysis after hydrolysis showed it to be pure. This peptide was not used in subsequent experiments and is included in Table 2 for comparison only.

III. RESULTS AND DISCUSSION

To determine whether differences in reactivity of wool cystine with oxidizing agents could be attributed to its different mode of linkage in the peptide chain, the following representative peptides were studied:

(1) Bisglycyl-L-cystine·

$$\left[\begin{array}{c} COOH \\ | \\ H_2N\ CH_2.CO.NH\ CH.CH.S - \end{array} \right]_2$$

(1') Bis-DL-alanyl-L-cystine:

$$\left[\begin{array}{c} COOH \\ | \\ H_2N.CH(CH_3)CO\ NH.CH\ CH_2.S - \end{array} \right]_2$$

With the two peptides containing both cystine and tyrosine, quite different results were obtained (Figs. 7 and 8). Chlorine at pH 2 still reacted specifically with the disulphide bond, though it reacted with tyrosine to a greater extent than it did with the "indifferent" amino acids alanine and glycine. In the case of the three previously examined peptides for example, 10 mole ($2 \times$ theoretical) of chlorine at pH 2 attacked more than 95 per cent. of the cystine. In the tyrosine peptides this value has dropped to 83 per cent. The reaction with hypochlorite at pH 10 however was completely different to that occurring with the other peptides so far examined. At low chlorine concentrations, the reaction was quite specific
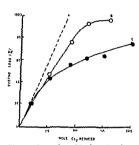
Fig. 4.—Loss of combined cystine from bisalanylcystinylbisglycine with chlorine oxidation.

A, Theoretical curve B, At pH 2. C. At pH 10.
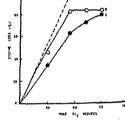
Fig. 5.—Loss of combined "cystine" from glutathione with chlorine oxidation.

A, Theoretical curve B, At pH 2. C. At pH 10.

for cystine, and 1 mole (20 per cent.) of chlorine removed 22 per cent. of cystine. At about 2.5 mole (50 per cent.) of chlorine however, the curve begins to flatten out and the difference in cystine loss between 2.5 mole and 10 mole of chlorine is very small. Only 45 to 50 per cent. of the cystine was attacked at the higher concentration.
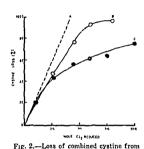
While, therefore, both peptides reacted initially so that quantitative amounts of cystine were oxidized, the product which was formed during the reaction inhibited further attack of the hypochlorite on the cystine. In a solution containing equimolar quantities of cystine and tyrosine, there was preferential oxidation of the cystine, both at pH 2 and pH 10 (see Fig. 9); even when the molar ratio of tyrosine to cystine was raised to 3 : 1, the hypochlorite still reacted preferentially with the cystine (see Table 3). The incomplete reaction of cystine in these peptides with chlorine at pH 10 arises, therefore, from its combination with tyrosine and is not due merely to the presence of this amino acid.

The predominant oxidation undergone by cystine is the formation of the sulphonic acid:

$$R.SS.R. + 5Cl_2 + 6H_2O = 2RSO_3H + 10HCl,$$

that is, 5 mole of chlorine is required for the complete oxidation of 1 mole of cystine to cysteic acid.

Figures 2 to 5 show that whereas both chlorine and hypochlorite oxidize all the disulphide bonds in bisglycylcystine, cystinylbisglycine, bisalanyl-cystinylbisglycine, and glutathione, only the first 20 per cent. of cystine

Fig. 2.—Loss of combined cystine from glycylcystine with chlorine oxidation.

A, Theoretical curve B, At pH 2.
C, At pH 10.
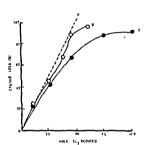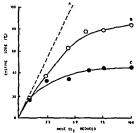
Fig. 3.—Loss of combined cystine from cystinylbisglycine with chlorine oxidation.

A, Theoretical curve B, At pH 2.
C, At pH 10.

is oxidized specifically at pH 2 and 10. Inspection of Figures 2 to 5 shows that oxidation of the disulphide bond by both chlorine and hypochlorite predominates up to concentrations which are sufficient to oxidize more than half the available disulphide bonds. Chlorine at pH 2 is somewhat more specific. The theoretical curves refer to specific oxidation of the cystine to the sulphonic acid.

The rate of disappearance of hypochlorite at pH 10 in reaction with peptides 1, 2, and 3 was also studied (the reaction at pH 2 was too rapid in all cases) using sufficient chlorine to oxidize 90 per cent. of the cystine. Figure 6 shows that there is no significant difference in rate between the different peptides. These two series of experiments demonstrate therefore that the reactivity of the disulphide bond is the same in peptides whether the cystine is bound through the NH₂-group, or the COOH-group, or through both.

tyrosine and large quantities are therefore required to oxidize the disulphide bond but surprisingly there is little difference between acid and alkaline solution.

Fig. 7.—Loss of combined cystine from bistyrosylcystine with chlorine oxidation.

A, Theoretical curve B, At pH 2.
C, At pH 10.

Fig. 8.—Loss of combined cystine from cystinylbistyrosine with chlorine oxidation.

A, Theoretical curve B, At pH 2.
C, At pH 10.

Fig. 9.—Loss of cystine from a mixture (1 : 1 mole) of cystine and tyrosine with chlorine oxidation.

A, Theoretical curve. B, At pH 2. C, At pH 10.

It is interesting to note that in wool the cystine residue is oxidized by chlorine and hypochlorite at a rate only slightly greater than the tyrosine (e.g. 10 g chlorine/100 g wool at pH 2 oxidize 40 per cent. of the cystine and 27 per cent. of the tyrosine). In mixtures of tyrosine and cystine (see Fig. 9 and Table 3), the latter is oxidized preferentially. This difference is probably due to the fact that the rate of the actual chemical reaction does not determine the rate of reaction in the fibre which is diffusion-controlled (Alexander, Gough, and Hudson 1951).
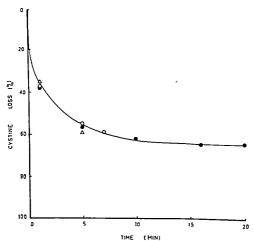
Fig 6.—Rate of loss of combined cystine from three peptides oxidized with 4.5 mole Cl_2/mole cystine.

● Bisalanylcystine (peptide 1).   ○ Cystinylbisglycine (peptide 2).
△ Bisalanylcystinylbisglycine (peptide 3).

(b) Reaction with Permanganate

Permanganate in acid solution rapidly and preferentially oxidizes the disulphide bond in the peptides (Table 4) which do not contain tyrosine. At pH 9.2 the reaction is much less specific and a considerable excess of permanganate is required to oxidize all the combined cystine.

The behaviour of the tyrosine peptides is again quite different. Both acid and alkaline solutions of permanganate react preferentially with

Cystine oxidation with permanganate is also different in the two groups of peptides; in the non-tyrosine peptides cystine is preferentially oxidized by permanganate in acid solution, but at pH 9.2 other reactions occur and a proportionally greater amount of oxidizing agent has to be used to obtain a similar degree of oxidation. In the tyrosine peptides cystine is not attacked preferentially either in acid or alkali, but with large excess of reagent it can be oxidized to roughly the same extent in acid and alkali.

TABLE 4

CYSTINE LOSS IN PEPTIDES AFTER TREATMENT WITH PERMANGANATE

Substrate	KMnO₄ Mole/Mole Combined Cystine	Disulphide Loss (%)
Cystinylbisglycine	2.4 at pH 2	96.2*
	4.0 at pH 9.2	70.1
	6.66 at pH 9.2	88.1
BisGlycylcystine	2.4 at pH 2	95.0
	4.0 at pH 9.2	77.6
	6.66 at pH 9.2	92.5
BisAlanylcystinylbisglycine	2.4 at pH 2	97.5
	4.0 at pH 9.2	52.2
	6.66 at pH 9.2	74.0
BisTyrosylcystine	2.4 at pH 2	32.8
	4.0 at pH 2	61.6
	4.0 at pH 9.2	38.0
	6.66 at pH 9.2	53.8
Cystinylbistyrosine	2.4 at pH 2	14.4
	4.0 at pH 2	42.7
	4.0 at pH 9.2	48.9
	6.66 at pH 9.2	58.2
Cystine-tyrosine mixture (1 : 1 mole)	2.4 at pH 2	16.5
	4.0 at pH 2	41.7
	4.0 at pH 9.2	35.2
	6.66 at pH 9.2	59.0

* Theoretically 2 mole permanganate at pH 2 and 3.33 mole at pH 9.2 are required to oxidize 1 mole cystine to cysteic acid

Clearly these experiments do not allow one to identify the less reactive (25 per cent.) fraction in wool with cystine linked to tyrosine, the remainder being linked to other amino acids. On the other hand, it is clear that whereas the mode of incorporation of cystine into simple peptides does not determine its reactivity with oxidizing agents, the proximity of a tyrosine residue does. We believe this to be of some relevance to wool, since both these amino acids show similar variations in reactivity. It is not to be expected that one can obtain complete correlation between the

(c) *Products of Oxidation*

In order to characterize the oxidation products the peptides were treated with peracetic acid to form the corresponding cysteic acid peptides. In the case of cystinyldiglycine and diglycylcystine the R_F value of the oxidation products was checked with that obtained by Consden and Gordon (1950). The specificity of peracetic acid for the disulphide bond was demonstrated when paper chromatograms of glycyltyrosine, tyrosylglycine, glycyltyrosylglycine, and the γ-methyl ester of glutamylglycylglycine, after treatment with this reagent, revealed only spots due to the original peptide.

Chromatograms of cystine peptides oxidized with chlorine revealed spots in addition to those due to the original peptide and the cysteic acid

TABLE 3

CYSTINE LOSS OF TYROSINE-CYSTINE PEPTIDES AND TYROSINE-CYSTINE MIXTURES

Treatment with 7.5 mole chlorine per mole cystine at pH 10

Substrate	Cystine loss %
Bistyrosylcystine	45.0
Cystinylbistyrosine	48.5
Tyrosine-cystine (1 : 1 mole) mixture	85.9
Tyrosine-cystine (3 : 1 mole) mixture	61.5

analogue. It is unlikely that they were due to intermediate cystine oxidation products when a substantial excess of oxidizing agent was used, and they are probably due to reaction with other centres in the peptide.

IV. CONCLUSION

If the difference in reactivity with oxidizing agents of the disulphide bonds in wool were related to their method of incorporation in the peptide chain one might ideally expect to find two types of cystine peptide: (1) Those (25 per cent. in wool) in which the cystine is oxidized by acid chlorine, hypochlorite, and acid permanganate; (2) those (75 per cent. in wool) which are oxidized by chlorine at pH 2, but only very slowly or not at all by hypochlorite and permanganate. Both groups should react only very slowly with alkaline permanganate which only oxidizes wool-cystine after extensive reaction with other centres. The cystine peptides studied do not fall into two such clear-cut classes but there are nevertheless differences amongst them.

The cystine was equally readily oxidized in all peptides not containing tyrosine. Incorporation of this residue led to incomplete oxidation by high concentrations of hypochlorite. The initial complete cystine oxidation with low hypochlorite concentrations appears to form some product which "protects" the remaining unreacted disulphide bonds.

MACROMOLECULAR STRUCTURE OF WOOL : THE TERMINAL AMINO ACIDS OF THE FRACTIONS OBTAINED AFTER OXIDATION WITH PERACETIC. ACID

By P. ALEXANDER* and L. F. SMITH†

Summary

The preparation of α-, β-, and γ- keratoses by treating wool with dilute peracetic acid and extracting with ammonium hydroxide is described. The N-terminal amino acids of these protein extracts and fractions prepared from them are compared with those of whole wool using the DNFB reagent of Sanger. The average chain weight determined in this way is compared with molecular weights determined by physical methods. It is suggested that α-keratose is derived from the micelles or subfibrils, γ-keratose from the intermicellar substance which cements the whole together through disulphide bonds, and β-keratose from the cell membranes surrounding the cortical cells.

I. INTRODUCTION

One of the fundamental problems in the chemistry of wool is the characterization of the proteins which make up the bulk of the fibre. The morphology is known to be complex and even non-medullated fibres consist of a very large number of individual cells of several clearly distinct types. These cells were at one time alive, with an active cytoplasm and nucleus containing a full complement of enzymes, structural proteins, nucleic acids, fats, minerals, and metabolites. All these substances must be present in the wool fibre, although undoubtedly only in small quantities since during the course of their development the hair cells become filled with the specialized proteins which eventually harden into keratin when the cells die. There can be no doubt that this special material completely swamps the cells and eventually comprises about 90 per cent. of their mass. The nuclei of the cells can be clearly seen as black spots in the "membrane" which remains behind after peracetic acid–ammonia extraction and which Mercer (1953) has shown to be made up of the membranes surrounding the individual cells. The whole cycle of the biosynthesis of wool starting from ordinary cells substantially free from keratin to the final "fossilized" cell of keratin has been revealed by Mercer (unpublished) in an electron microscopic investigation.

In this paper we are concerned only with the different proteins which make up the keratin filling the cells. This material is present in the form of fibrils, the existence of which was first revealed by micro-manipulation under the light microscope by Hock, Ramsay, and Harris (1941). These fibrils can be broken down further into subfibrils visible only in the

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behaviour of cystine in simple peptides in solution and in wool where the
reaction is hetereogenous and the rate process is diffusion-controlled.

V. REFERENCES

ALEXANDER, P., and GOUGH, D. (1951).—*Biochem. J.* 48: 504.
ALEXANDER, P., GOUGH, D., and HUDSON, R. F. (1951).—*Biochem. J.* 48: 20.
ALEXANDER, P., HUDSON, R. F., and FOX, M. (1950).—*Biochem. J.* 46: 27.
BAILEY, J. L. (1950).—*J. Chem. Soc.* 1950: 3461.
CONSDEN, R., and GORDON, A. H. (1950).—*Biochem. J.* 46: 8.
FRASER, R. D. B., LINDLEY, H., and ROGERS, G. E. (1954).—*Biochim. Biophys. Acta* 13:
    295.
HORIO, M., and KONDO, T. (1953).—*Text. Res. J.* 23: 373.
MERCER, E. H. (1953).—*Text. Res. J.* 23: 388.
MERCER, E. H. (1954).—*Text. Res. J.* 24: 195.
PHILLIPS, H. (1946).—"Symposium on Fibrous Proteins." pp. 39 *et seq.* (Soc. Dy. Col.:
    Bradford.)
SCHURINGA, G. J. (1950).—*Text. Res. J.* 20: 370.
SHINOHARA, K. (1935).—*J. Biol. Chem.* 112: 671.

γ-keratose acts as an intermicellar cement (a cement between the sub-fibrils) from the fact that this material is the first to be extracted from partially oxidized fibres.

II. EXPERIMENTAL

(a) Preparation of Wool Fractions

Wool fabric knitted from a high quality Botany yarn, which had received no prior treatment other than mild scouring with ammonia to remove wool grease and dirt, was washed and extracted in a Soxhlet with ethanol and ether, and air dried. The wool was oxidized with a 1.6 per cent. (w/v) solution of peracetic acid (liquor/wool ratio 50:1) which had been diluted from a 45 per cent. (w/v) stock solution prepared by the method of Greenspan (1946) from 90 per cent. H_2O_2. After 25 hr, during which it had been shaken at intervals, the wool was thoroughly washed in distilled water and air dried. The peracetic acid contains H_2SO_4 which tends to be bound to the wool, giving erroneously high values for the sulphur content of the fractions. It is of the greatest importance to ensure that all SO_4 ions are removed from the oxidized wool by washing before proceeding to the next stage. After this the oxidized wool was dissolved in either 3N or 0.1N NH_4OH for 12 hr, using a liquor ratio of 200:1. On shaking, the fibres slowly disintegrated, giving a viscous solution. The residue (β-keratose) was removed by centrifugation and washed repeatedly with dilute NH_4OH until no precipitate was formed on acidifying a portion of the supernatant liquor. Finally, it was washed free from alkali and dried in a vacuum desiccator.

The brown viscous supernatant liquor still contained very finely divided material in suspension and this was removed by filtering, firstly, through a No. 4 sintered funnel and, secondly, through a porcelain filter candle of pore size 0.2 μ. Excess ammonia was removed under vacuum and the solution was brought to pH4 by the addition of HCl and the white curd-like precipitate centrifuged off and washed with distilled water until free from Cl⁻ ions. This fraction was called "whole α". The supernatant liquor contained NH_4Cl and protein material soluble in both acid and alkali and this fraction was called the "γ" fraction. It could not be precipitated from solution by acetone or trichloroacetic acid. The γ-keratose together with a quantity of NH_4Cl was obtained by freeze drying the solution. The protein content (of the order of 50 per cent.) was determined by analysing the solid for chloride, which gave the amount of NH_4Cl present. If the H_2SO_4 in the peracetic acid was not completely removed it appeared in this fraction as $(NH_4)_2SO_4$ which could readily be detected with $BaCl_2$ which does not precipitate the cysteic acid peptides. From a number of preparations the yield for α, β, and γ-fractions were 60, 10, and 30 per cent. respectively.

(i) *Further Fractionation of α-Keratose.*—An attempt was made to fractionate α-keratose by fractional precipitation from concentrated urea

electron microscope. Immediately after its formation the keratin can be dispersed in solvents that break hydrogen bonds but in the fibre itself it is completely insoluble, having become hardened by the formation of disulphide cross-links into the complex entity referred to as keratin.

To study the macromolecular constitution of keratin it is therefore necessary to break the disulphide bonds by a reagent which does not produce main-chain degradation by attacking peptide bonds. Further, to render the resulting products soluble in water near the neutral point, it is necessary to introduce additional ionizing groups. For example, reduction of the disulphide bond to two sulphydryl groups does not render keratin soluble in water at pH 7, the reduced product will dissolve only in alkaline solution when the —SH group becomes ionized or in solvents, such as concentrated urea solutions, that break hydrogen bonds. Sanger (1948) solved a similar problem by using performic acid to oxidize the disulphide bonds joining the polypeptide chains of insulin. This reagent converts the disulphide bond into two sulphonic acid groups

$$R—CH_2—S—S—CH_2—R \rightarrow 2R—CH_2SO_3H,$$

without attacking the peptide bond; the only other amino-acid residues attacked are those from tryptophane and methionine. Performic acid, however, is an unstable material which decomposes when diluted with water and is not very suitable for treating wool that is severely modified by the concentrated formic acid in which the performic acid is dissolved. A much more suitable reagent was found to be peracetic acid (Alexander, Hudson, and Fox 1950) which is relatively stable even in very dilute aqueous solution so long as the pH is less than 7. In its chemical reactions it is as specific as performic acid but in dilute aqueous solutions will be much less harmful than performic acid dissolved in concentrated formic acid.

After oxidation of all the disulphide bonds with peracetic acid, 90 per cent. of the wool becomes soluble in solutions of pH > 8 and in this way it has been separated (Alexander and Earland 1950; Alexander 1951) into a number of fractions which are referred to as keratoses since they are derived from oxidized keratin. The β-keratose was believed by us to consist of a membrane originally situated between the scales and the cortex but Mercer (1953) showed that it was in fact a honeycomb structure made up of the membranes from all the cells which make up the fibre. From study of the mechanical properties and crystalline structure of wool that has been treated with peracetic acid, and in particular from its super-contraction behaviour, we deduced that the fibrils were made up of aggregates of high molecular weight proteins (α-keratose) set in a cement of the cystine-rich proteins of lower molecular weight (γ-keratose).

In earlier publications we reported the physical properties of these different fractions (cf. Alexander and Hudson 1954); their characterization by determination of the N-terminal amino acids forms the subject of this communication. Further evidence is adduced for the hypothesis that

acid, and water. The weight increases of wool and β-keratose were 10 and 8 per cent. respectively. Only about 80 per cent. of the α-keratose was precipitated as a result of the DNFB treatment, while almost all the γ-keratose remained in solution. The precipitates were not separated and the mixture treated as a whole. The latter was therefore treated as if it were made up of soluble DNP proteins. The ethanol was removed by evaporation under vacuum at 40°C and unreacted DNFB was extracted with ether. By using the continuous extraction apparatus designed by Mills (1952) the problem of breaking the stable emulsion between ether and the protein solution was overcome. The mixture was acidified with dilute HCl when the dinitrophenol formed by hydrolysis of DNFB could also be extracted with ether. The residual solution and precipitate was evaporated to dryness under vacuum. The DNP proteins were hydrolysed by Middlebrook's (1951) procedure by refluxing for 24 hr with 5.7N HCl.

Separation of DNP Amino Acids.—After hydrolysis the solution was filtered, diluted with water, and extracted with ether until the ether layer remained colourless. The extract containing the α-DNP amino acids as well as dinitrophenol and dinitroaniline from the breakdown of DNP amino acids, was washed with a small quantity of 1N HCl and then extracted with a 1 per cent. solution of $NaHCO_3$. Only the DNP amino acids and dinitrophenol went into the $NaHCO_3$ solution, which was acidified, re-extracted with ether and the ether-soluble material taken to dryness. This material was then used directly for fractionation, which was carried out on buffered columns of a kieselguhr marketed as Celite 545, ethyl acetate being used as the mobile phase to give much better separations than ether or chloroform. All the end-groups in wool except DNP-valine could be separated on two columns. DNP-valine could not be separated from dinitrophenol on ethyl acetate columns. This separation was achieved by using a chloroform column at pH 6.4. The acid soluble DNP amino acids were fractionated on columns buffered at pH 6.4 using water-saturated 30 per cent. butanol-chloroform as the mobile phase. When the DNP-valine plus dinitrophenol band had been eluted and taken to dryness it was not very soluble in chloroform. It was found that if the residue was acidified with a drop of concentrated HCl and some solvent added, the whole being taken to dryness, then the band would dissolve readily in the solvent once more.

The ether soluble DNP amino acids were first fractionated on an ethyl acetate column pH 7.4, when the following bands were obtained:

	R_f*
DNP-aspartic acid, DNP-glutamic acid	
DNP-serine, DNP-threonine	Slow
DNP-glycine	0.25
DNP-alanine	0.4
DNP + DNP-valine	1.0
Artefact 1	1.4
DN-aniline	1.6

* R_f = Distance travelled by band/distance travelled by liquid surface.

solution. Whole α-keratose (8 g) was dissolved in 450 ml of saturated urea solution. The solution was brought to neutrality with 2N H_2SO_4 and 11 ml of 2N H_2SO_4 added in excess. Water (330 ml) was added and the precipitate filtered off; the filtrate was diluted to 2,000 ml and allowed to stand overnight after the addition of 27 ml of H_2SO_4. A further precipitate (first α) was filtered off and washed. The first precipitate was treated again in the same way, the first precipitate being refractioned and the second precipitate (second α) washed. This was repeated two more times, the first precipitate of the last fractionation (fifth α) being collected. The fifth α fraction would be the high molecular weight material and the first α the low molecular weight material if whole α-keratose were not homogeneous.

(ii) *Extract from Partially Oxidized Wool.*—Alexander, Fox, and Hudson (1951) studied the relation between the amount of cystine oxidized in the fibre and the solubility of the fibre in dilute alkali. The shape of the graph obtained varied with the oxidizing agent used. With peracetic acid the solubility remained constant at about 10 per cent., while the amount of cystine oxidized was increased from 20 to 75 per cent. When more than 75 per cent. of the cystine was oxidized the solubility increased rapidly to 90 per cent. The shape of the curve using peracetic acid suggests that there is an initial stage when part of the fibre is preferentially oxidized, after which a more random oxidation takes place.

The wool fabric was oxidized by shaking for 0.5 hr at room temperature with a 1.6 per cent. solution of peracetic acid (liquor ratio 50:1) to oxidize about 50 per cent. of the cystine. It was then cut up into small pieces and treated with 0.1N or 3N NH$_4$OH overnight. The mixture (containing about 90 per cent. of the fibres undissolved) was placed in a Waring blendor, homogenized, filtered, re-extracted in the blendor and filtered again. Excess NH$_4$OH was removed from the filtrate under vacuum. On acidification to pH 4 a faint precipitate was formed, which was centrifuged off. The supernatant solution was freeze-dried. The protein content of this material represented 7 per cent. of the original wool.

(b) *Determination of Terminal Amino Groups*

Wool or the wool fractions (0.5 g) was treated with a solution made up of 0.5 g NaHCO$_3$ in 12.5 ml water and 0.5 ml dinitrofluorobenzene (DNFB) in 25 ml of ethanol. The absolute quantities used depended on the amount of protein required to give a sufficient quantity of DNP amino acids to separate on the columns. With whole wool this was about 1.5 g while for the lowest molecular weight fractions only 0.3 g was necessary. Wool and the insoluble β-fraction were treated for 2 days at 40°C with intermittent shaking. To the α- and γ-fractions dissolved in the bicarbonate solution DNFB in ethanol was added and shaken for 2 hr at 40°C. Excess DNFB as well as its hydrolysis products were removed from ordinary wool and β-keratose by washing with ethanol, dilute acetic

wool was measured by Middlebrook (1951). The value for DNP-glycine was determined by Sanger (1945).

III. RESULTS

(a) *End-groups in Whole Wool*

The end-groups of whole wool were determined, using the DNFB method, by Middlebrook (1951) who found an average chain weight (ACW) (i.e. the average size of polypeptide chains terminated by an amino group) of 59,000 and a total of seven N-terminal amino acids. We found the same N-terminal amino acids present in approximately the same ratio but their total quantity gave an ACW of 86,000 (see Table 1).

TABLE 1

N-TERMINAL AMINO ACID COMPOSITION OF WHOLE WOOL
Mole/10^6 g protein

Terminal Amino Acid	Duplicate Estimations		Average
Aspartic acid	0.6	0.4	0.5
Glutamic acid	1.0	0.8	0.9
Serine	1.1	1.0	1.05
Threonine	3.3	3.1	3.2
Glycine	4.1	3.5	3.8
Alanine	1.1	1.1	1.1
Valine	0.95	1.0	1.0
Total	12.15	10.9	11.55
ACW			86,000

This discrepancy in ACW is not as serious as it appears since some of the DNP amino acids are destroyed in part during the hydrolysis and this loss is corrected by experimentally-determined values for the percentage decomposition. Such values are likely to be sensitive to slight differences in the conditions of hydrolysis.

(b) *End-groups in α-Keratose*

Two kinds of preparation were studied, in which the oxidized wool was extracted with 3N NH$_4$OH (referred to as α—3N) or else with 0.1N NH$_4$OH (α—0.1N). As can be seen from Table 2 there was no significant difference either in the total number of end-groups (i.e. in the ACW) or in their relative proportions.

The reproducibility of the results was satisfactory and in a number of independent determinations the variations for the individual N-terminal end-groups did not exceed 15 per cent. and the variation in ACW was considerably less. A notable feature of these results (this applies also to the end-group analyses of the β- and γ-fractions) is that the same seven

The bands comprising the DNP derivatives of glycine, alanine, and valine (with DNP) were collected separately and DNP derivatives of aspartic acid, glutamic acid, serine, and threonine eluted by adding a small amount of glacial acetic acid to the eluant.

The dinitrophenol and DNP-valine were fractionated on a chloroform column at pH 6.4 giving these bands:

	R_F
DNP-valine	0.8
Artefact 2	1.1
Dinitrophenol	1.4.

The DNP derivatives of aspartic acid, glutamic acid, serine, and threonine, which were separated on an ethyl acetate column buffered at pH 5.9, moved as follows:

	R_F
DNP-aspartic acid	0.1
DNP-glutamic acid	0.25
DNP-serine	0.6
DNP-threonine	0.9.

The acid soluble DNP derivatives were fractionated on 30 per cent. butanol-chloroform columns at pH 6.4 giving:

	R_F
DNP-lysine	0.8
Artefact 3	1.5 to 1.8.

After elution, the material from the different bands was freed from solvents by evaporation to dryness and was redissolved in 1 per cent. NaHCO$_3$ for estimation in a spectrophotometer at 350 mμ (Sanger 1949). As shown in the tables above, three bands were obtained which could not be accounted for as known DNP amino acids. Two tests were applied to these bands to show that they were not DNP amino acids, (a) the ultraviolet absorption spectra of 2 and 3 were not those of typical DNP amino acids; (b) Lowther (1951) had shown that the original amino acid could be regenerated from a DNP amino acid by heating with concentrated NH$_4$OH for 2 hr at 100°C. When these bands were treated in this manner and run on chromatograms, no amino acids were obtained. Blackburn (1949) had earlier noted the presence of artefacts in hydrolysates of DNP wool. The artefact bands 1 and 2 may be produced by non-keratin material originating from the cellular material such as nucleic acids and carbohydrates.

The acid-soluble fraction was investigated qualitatively only, to see if additional bands appeared during the fractionation. The liquors in which the wool and wool fractions were treated were examined for free DNP amino acids, but none were detected.

The DNP amino acids are partially destroyed during the hydrolysis of the DNP protein and corrections had to be applied to the final results to allow for this. The breakdown of the DNP amino acids present in DNP

0.1N NH₄OH. This difference is accounted for by an almost fourfold increase in DNP-serine in the preparation obtained by using the more concentrated alkali.

The increase in DNP-serine in the γ-fraction prepared using strong NH₄OH can occur in two ways:

(i) A low molecular weight peptide having an *N*-terminal serine residue may be split from chains in the α- and β-fractions. This polypeptide with the *N*-terminal serine then passes into the γ-fraction.

$$
\begin{array}{ccc}
\text{CH}_2\text{OH} & \text{R}' & \text{R}'' \\
| & | & | \\
\text{NH}_2-\text{CH CO} \ldots\ldots\ldots\ldots \text{NH CH CO NH CH CO} \ldots
\end{array}
$$

$$\downarrow$$

$$
\begin{array}{ccc}
\text{CH}_2\text{OH} & \text{R}' & \text{R}'' \\
| & | & | \\
\text{NH}_2-\text{CHCO} \ldots\ldots\ldots\ldots \text{NH CH CO}_2\text{H} + \text{NH}_2 \text{CH CO} \ldots
\end{array}
$$

(ii) A peptide bond in which the amino group of a serine residue is involved is broken by the strong ammonia. The peptide undergoing hydrolysis in this way would either have to be in the γ-fraction or, if in the α- and β-fractions, to be of low molecular weight and to pass into the γ-fraction after hydrolysis.

$$
\begin{array}{ccc}
\text{R} & \text{CH}_2\text{OH} & \text{R} \\
| & | & | \\
\ldots \text{NH CH CO NH CH CO} \ldots\ldots \text{NH CO CH NH}_2
\end{array}
$$

$$\downarrow$$

$$
\begin{array}{ccc}
\text{R} & \text{CH}_2\text{OH} & \text{R} \\
| & | & | \\
\ldots \text{NH CH CO}_2\text{H} + \text{NH}_2 \text{CH CO} \ldots \text{NH CO CH NH}_2
\end{array}
$$

The first mechanism can be eliminated since the numbers of *N*-terminal serine residues in α- and β-keratose, either alone or combined, are insufficient to cause the observed increase.

To establish in which of the wool fractions the ammonia produced the peptide bond breakdown at the serine residue, whole wool as well as α-, β-, and γ-keratose isolated with 0.1N NH₄OH were treated for 12 hr at room temperature with 3N NH₄OH before determining their DNP end-groups in the usual way. Treatment with NH₄OH of intact wool as well as of the fractions isolated with 0.1N NH₄OH did not produce any increase in DNP-serine and the end-group analysis is within experimental error unchanged (the values for the γ-fraction are given in Table 3).

It is well known that the peptide bond formed by the α amino group of serine is much weaker than the peptide bonds formed by other amino acids. Desnuelle and Casal (1948) found that when proteins were treated with 10N HCl at 30°C, 20 to 30 per cent. of the serine and threonine α-amino groups were liberated after 1 hr. Only very few other amino groups appeared. At lower temperatures the reaction was still more specific. Unfortunately no comparable data is available for alkaline

amino acids are present as end groups in the different fractions as in whole wool although in different proportions and total amounts.

In earlier publications (Alexander 1951) we have reported that α-keratose was not electrophoretically homogeneous but contained a small proportion of a slower moving component of lower molecular weight which could be removed by precipitation from urea solution. In the present preparations no such component was found when the initial preparations were carried out slowly and the contaminant was probably part of the γ-keratose fraction brought down with the main α-fraction. The end-group analysis shows that α-keratose giving a single electrophoretic peak is not homogeneous and a fractionation is possible. The analysis shown in Table 2 establishes that some separation has been achieved and the first and last

TABLE 2

N-TERMINAL AMINO ACID COMPOSITION OF THE α- AND β-KERATOSE FRACTIONS OF WOOL
Mole/10^6 g of protein

Terminal Amino Acid	β-3N	β-0.1N	α-3N	α-0.1N	1st α-Fraction	5th α-Fraction
Aspartic acid	1.3	1.2	0.9	1.05	1.0	1.2
Glutamic acid	3.1	3.4	2.9	2.5	2.1	2.1
Serine	2.8	2.7	2.0	2.1	2.7	1.8
Threonine	1.2	0.8	2.15	2.4	2.7	1.5
Glycine	8.75	10.0	5.8	5.7	7.6	5.0
Alanine	2.8	2.6	1.9	2.1	1.7	1.4
Valine	1.9	2.1	1.2	1.2	0.45	0.6
Total	21.85	22.8	16.85	17.05	18.25	13.6
ACW	45,700	43,900	59,400	58,600	54,800	73,500

fractions differ significantly in their terminal amino acids although the procedure clearly does not result in the isolation of a uniform material. The fifth fraction would be expected to be of higher molecular weight and this is borne out by the ACW.

(c) End-groups in β-Keratose

There is no significant difference between the extraction of β-keratose with 0.1N and 3N NH₄OH (Table 2). With the dilute ammonia however the insoluble β-keratose cannot be freed completely from soluble material and as much as 14 per cent. of the original wool may be present in this fraction. With 3N NH₄OH this is reduced to 10 per cent. The contaminants of the β-0.1N give rise to soluble DNP products which are not included in the final analysis.

(d) End-groups in γ-Keratose

Table 3 shows that, unlike α- and β-keratose, there is a profound difference in the ACW of the γ-keratoses obtained by solution in 3N and

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The end-group distribution of γ_1 and γ_2 does not differ significantly from that found in the unfractionated γ-keratose and the increased serine content when 3N NH$_4$OH was used is evident in both subfractions (Table 3). The major difference between γ_1 and γ_2 is in their sulphur content (Table 5).

TABLE 4
N-TERMINAL AMINO-ACID COMPOSITION OF EXTRACT FROM PARTIALLY OXIDIZED WOOL
Mole/10^6 g protein

Terminal Amino Acid	Extracted with	
	3N NH$_4$OH	0.1N NH$_4$OH
Aspartic acid	2.7	
Glutamic acid	6.55	2.6
Serine	13.4	6.0
Threonine	10.0	9.4
Glycine	20.2	11.4
Alanine	6.45	24.2
Valine	6.4	8.55
Total	65.7	5.3
ACW	15,200	67.45
		14,800

(e) Extract from Partially Oxidized Wool

Although the end-group distribution and ACW of this material is different from all the wool fractions (Table 4) it bears a similarity to γ-keratose and in particular to its dialysable component γ_1.

TABLE 5
ELEMENTARY ANALYSIS OF THE DIFFERENT FRACTIONS AND SUBFRACTIONS OBTAINED FROM PERACETIC ACID OXIDIZED WOOL

Wool Fraction	C	H	N	S
Wool	48.7	7.04	16.32	
α-Keratose				
1st Urea fraction	48.8	6.73	15.95	3.63
5th Urea fraction	46.5	6.79	14.78	2.5
β-Keratose	45.6	6.97	14.88	2.48
γ-Keratose (3N NH$_4$OH)	50.4	6.99		2.24
γ$_1$ (dialysable)	45.3		15.40	2.2
γ$_2$ (non-dialysable)	41.4			6.13
Extract from partially oxidized wool (0.1N NH$_4$OH)	42.8			7.6
	45.6			4.55
				5.6

The DNP-serine content depends on the strength of the NH$_4$OH used for the extraction although the effect is less marked than in the γ-fraction.

hydrolysis but it may be that the peptide bond adjacent to a serine residue is labile under these conditions also. A possible explanation for the ability of NH_4OH to liberate certain serine end groups in oxidized wool only and not in the fractions isolated from oxidized wool may be found in the observation by Alexander, Fox, and Hudson (1951) that the disulphide bond is oxidized in the fibre to an unstable cyclic sulphonamide, this ring opening during the dissolution process to give cysteic acid. This reaction may alter the susceptibility of the peptides to hydrolysis.
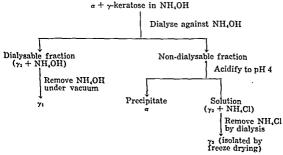
TABLE 3

N-TERMINAL AMINO ACID COMPOSITION OF THE γ-KERATOSE FRACTION OF WOOL

Mole/10^6 g of protein

Terminal Amino Acid	γ-3N	γ-0.1N	γ-0.1N treated with 3N NH_4OH for 12 hr	$γ_1$-3N	$γ_2$-3N	$γ_2$-0.1N
Aspartic acid	1.5	1.3	1.4	2.0	2.8	1.4
Glutamic acid	4.3	5.2	5.7	6.55	7.6	5.8
Serine	14.8	4.4	3.6	13.05	14.7	4.3
Threonine	8.3	7.45	8.7	8.75	9.4	6 25
Glycine	10.4	8.6	7.1	12.6	Lost	9.6
Alanine	5.5	4.45	4.0	6.95	Lost	4.9
Valine	4.8	3.9	4.8	6.3		3.3
Total	49.6	35.3	35.3	56.2		35.55
ACW	20,200	28,300	28,300	17,800		28,100

A partial fractionation of γ-keratose was obtained by dialysis through cellophane tubing obtained from the Visking Corporation, U.S.A., as follows:

α + γ-keratose in NH_4OH

Dialyse against NH_4OH

Dialysable fraction ($γ_1$ + NH_4OH)

Remove NH_4OH under vacuum

$γ_1$

Non-dialysable fraction

Acidify to pH 4

Precipitate α

Solution ($γ_2$ + NH_4Cl)

Remove NH_4Cl by dialysis

$γ_2$ (isolated by freeze drying)

3 to 14 residues/10^5 g. There seems to be no reason why this low value should not be real, since whole wool and the two other fractions both have N-terminal serine residues.

The total number of end groups found in the fractions (Table 6) is very nearly double that found by us in intact wool (Table 1). It is however most unlikely that this is due to peptide bond breakdown since no new end-groups are found in any of the fractions and, except for threonine, the end-groups are present in the same proportions as in whole wool. The most likely explanation would appear to be that a proportion of the end groups are sterically inaccessible to DNFB in the fibre itself. Thus we had earlier shown (Alexander 1951) that only 75 per cent. of the tyrosine hydroxyl groups present in the fibre could react with DNFB. This observation has been confirmed by Zahn and Würtz (1952) and extended to collagen. Porter (1948) first observed that in some native proteins only part of the ϵ-amino groups of lysine could react with DNFB and this has now been found to apply also to collagen and silk (Zahn and Wegerle 1954).

(b) Molecular Weight of the Subfractions

Physicochemical methods (Alexander 1951) gave a molecular weight (M_w) of 67,000. This value is a weight average whereas the ACW obtained in the present work gives a number average molecular weight (M_n). For a homogeneous material $M_w = M_n$. In heterogeneous materials $M_w > M_n$; for example a mixture containing a random distribution of molecules (as often found in synthetic macromolecules) $M_w = 2M_n$. The fractionation in urea (Table 2) proves that the polypeptides in α-keratose besides differing in end groups also vary in molecular weight. The fact that the ACW (59,000) is so close to the M_w of 67,000 shows that the variation cannot be very great and the range of 55,000 to 73,000 found in the fractionation by urea may well represent the extremes.

For the insoluble β-keratose the ACW represents the only information concerning the length of the chains and no comparison with other measurements is possible.

The end-group analysis for γ-keratose indicates an ACW of 29,100. There are reasons for believing that this is not the true molecular weight:

(i) It is readily dialysable. Only 18 per cent. remained as non-dialysable residue after three days. If it had a molecular weight of 29,000 it should be non-dialysable.

(ii) It cannot be precipitated from solution by acetone or trichloroacetic acid.

(iii) Diffusion experiments indicated that it is heterogeneous having a molecular weight less than 4,000.

(f) *Elementary Analysis of the Wool Fractions*

Alexander and Earland (1950) showed that the major part of the sulphur in wool was found in the γ-fraction as cysteic acid and that the α- and β-fractions contained a smaller percentage of sulphur than the original fibre. Table 5 summarizes the analysis obtained with the fractions studied in this paper.

The further fractionation of α- and γ-keratose has obviously achieved some separation and the material of lowest molecular weight γ_1, and of highest molecular weight, α (5th urea fraction), have the highest and lowest sulphur content respectively.

IV. DISCUSSION AND SUMMARY

(a) *Peptide Bond Breakdown during Fractionation*

To obtain information about the macromolecular structure of wool from the fractionation of oxidized wool it is necessary to establish that no

TABLE 6

END-GROUP COMPOSITION OF WHOLE WOOL AS DETERMINED FROM THE THREE KERATOSE FRACTIONS α, β, AND γ

Mole/10^6 g protein

Terminal Amino Acid	60%-α	10%-β	30%-γ	Total
Aspartic acid	0.58	0.127	0.375	1.1
Glutamic acid	1.62	0.347	1.59	3.6
Serine	1.2	0.28	1.185	2.7
Threonine	1.43	0.118	2.256	3.8
Glycine	3 28	0.888	2.676	6.8
Alanine	1.24	0.28	1.11	2.6
Valine	0.70	0.213	1.11	2.0
Total				22.6
ACW				44,200

peptide bond breakdown has occurred during any of the treatments. Sanger (1948) has shown conclusively that performic acid does not degrade insulin and it is therefore unlikely that peracetic acid used under much milder conditions will break peptide bonds. Direct experiments with synthetic peptides from a number of amino acids including cystine (Fox 1951) show that even prolonged treatment with peracetic acid and 0.1N NH₄OH does not break peptide bonds. Since the number of serine end groups in γ-keratose is increased by treatment with 3N NH₄OH the possibility remains that even the low serine end group value obtained in the 0.1N NH₄OH fractionation is not real but due to breakdown. Since this amounts to only 3 serine residues for every 10^6 g of γ-keratose, any such error if it existed would not be serious. The real damage occurs when the NH₄OH strength is increased to 3N and the end group increased from

VI. REFERENCES

ALEXANDER, P. (1951).—*Kolloidzschr.* 122: 8.

ALEXANDER, P., and EARLAND, C. F. (1950).—*Nature* 166: 396.

ALEXANDER, P., FOX, M., and HUDSON, R. F. (1951).—*Biochem. J.* 49: 129.

ALEXANDER, P., and HUDSON, R. F. (1954).—"Wool: Its Chemistry and Physics." (Chapman & Hall, Ltd.: London.)

ALEXANDER, P., HUDSON, R. F., and FOX, M. (1950).—*Biochem. J.* 46: 27.

BLACKBURN, S. (1949).—*Biochem. J.* 45: 579.

DESNUELLE, P., and CASAL, A. (1948).—*Biochim. Biophys. Acta* 2: 64.

GREENSPAN, F. P. (1946).—*J. Amer. Chem. Soc.* 68: 907.

FOX, M. (1951).—Ph.D. Thesis, University of London.

HOCK, C. W., RAMSAY, R. C., and HARRIS, M. (1941).—*J. Res. Nat. Bur. Stand.* 7: 181.

HORIO, M., and KONDO, T. (1953).—*Text. Res. J.* 23: 373.

LOWTHER, A. G. (1951).—*Nature* 167: 767.

MERCER, E. H. (1953).—*Text. Res. J.* 23: 388.

MIDDLEBROOK, W. R. (1951).—*Biochim. Biophys. Acta* 7: 547.

MILLS, G. L. (1952).—*Biochem. J.* 50: 707.

PORTER, R. R. (1948).—*Biochim. Biophys. Acta* 2: 105.

SANGER, F. (1945).—*Biochem. J.* 39: 507.

SANGER, F. (1948).—*Nature* 162: 491.

SANGER, F. (1949).—*Biochem. J.* 45: 563.

ZAHN, H., and WEGERLE, D. (1954).—*Das Leder* 5: 121.

ZAHN, H., and WURTZ, A. (1952).—*Biochem. Z.* 322: 324.

These discrepancies could be understood (*a*) if proline is an *N*-terminal residue since it would not be detected in the present work as DNP-proline is destroyed under the hydrolysis conditions used; (*b*) if part of the γ-fraction consists of cyclic polypeptides which have no terminal residues. A polypeptide mixture containing both open chain and cyclic molecules gives a higher ACW as estimated by the end group method than by physical methods.

The partial fractionation by dialysis into two fractions of different sulphur contents points to γ-keratose being heterogeneous, although the end group analysis of both fractions is almost the same.

(c) The Structure of Wool

Recent electron microscopic evidence has proved that β-keratose is present in the intact fibre as the cell membranes surrounding the cortical cells. Nothing in the present work is incompatible with the view deduced from experiments in supercontraction and permanent set (Alexander 1951) that α-keratose is derived from the micelles or subfibrils and γ-keratose from the intermicellar substance which "cements" the whole together through disulphide bonds. That the cysteine in wool is very irregularly distributed is emphasized by the fact that it has been possible to obtain a fraction (γ₁) containing as much as 7.6 per cent. sulphur. The observation that the extract from partially oxidized wool belongs to the sulphur-rich γ-keratose class provides further support for the above model. A reagent like peracetic acid specifically breaking disulphide bonds will attack the cement substance which would then become partly extractable. The micellar material would not be released, on this hypothesis, until the cement had been completely broken (i.e. the disulphide bonds fully oxidized) and this is in fact observed.

How far these results have to be reinterpreted since the discovery of the bilateral structure of wool by Horio and Kondo (1953) and Mercer (1953) cannot yet be decided. Since the same type of fractionation is obtained with keratin from horn it seems unlikely that the α- and γ-fractions originate from the different segments. The theory that the fractions arise intracellularly still allows for the difference in constitution observed between the *ortho-* and *paracortex* since these fractions are not homogeneous. An alternative view, consistent with all the facts, is that the ratio of α- to γ-keratose is different in the two segments; the *ortho-*cortex containing the higher proportion of cystine rich cementing substance giving rise to γ-keratose.
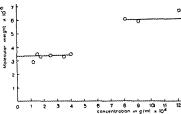
V. Acknowledgment

either herring sperm or calf thymus. There is, however, a marked difference between the two in the amount of residual protein

To show this effect clearly the concentration of versene required is 0·1 M which is the strength at which this and other chelating agents are effective in the method Kirby has developed for the separation of DNA from protein[5]. It is clear from the great difference in size, a factor over a hundred, that a trace of protein contaminant of this order, could provide sufficient polypeptide chains adhering to those of nucleic acid to inhibit the unfolding in urea. It seems possible therefore that the action of versene on DNA from calf thymus consists of the removal of this trace of protein impurity which may interfere with the disentangling process

The electron micrograph was taken on a Siemens electron microscope (Model UM 100) by Mr. M. S. C. Birbeck, for whose cooperation we are most grateful. We have developed a new technique for the preparation of DNA specimens which will be described in a future publication.

Fig. 2.—Molecular weight determined from light scattering data of DNA from herring sperm after standing at different concentrations in 4 M urea.

Fig. 3.—Electron micrograph of DNA from herring sperm; magnification 96,000. The width of shadow (platinum) of the thinnest fibres is 100 Å which corresponds to a fibre width of 20 Å under the condition of shadowing used. The numerals written in the fibres indicate the estimated number of double-helix threads which lie side by to make up the fibre

Zusammenfassung

DNA spaltet in 4-m-Harnstofflösung, wenn die DNA Konzentration weniger als 0,04% beträgt, in 2 g! grosse Teile auf. Elektronenmikroskopische Aufnahmen zeigen, dass das Ausbleiben einer Dissoziation in konzentrierten Lösungen durch P--- --- lagerung der Fäden bedingt sein durfte, eine Zusammenlagerung, welche den von W. Kuhn in der vorangehenden Mitteilung für die Langsteilung vorgeschlagenen Mechanismus behindern wurde.

5 K. S. Kirby, Biochem. J. (in press).

The Influence of Concentration on Dissociation of Deoxyribosenucleic Acid by 4 Molar Urea

In earlier publications[1] we had shown that the molecular weight of deoxyribosenucleic acid (DNA) as determined by light-scattering fell to one half its value in dilute salt solution when sufficient urea was added to bring the final concentration to 4 M. This and the associated changes in the shape of the molecule were consistent with the view that a longitudinal splitting of the double molecule had occurred. In all these experiments concentrated urea was added to solutions containing less than 0.04% of DNA since these were most convenient for light-scattering experiments. When urea was added to solutions containing more than 0.08% DNA dissociation no change was observed in molecular weight within 3 h (Fig. 1). Since it was not possible to measure molecular weights directly at these concentrations experiments were carried out as follows.

Equal volumes of the initial DNA solution and a solution of 8 M urea were mixed and allowed to stand. They were then diluted four times with dilute salt solution so that the final urea concentration was 1.0 M and the light scattering measurements made. A parallel experiment was made in which the final concentration of DNA and urea were the same but the dilutions were made in such a way that the DNA was never exposed to urea of more than 1.0 M. This concentration is not capable of dissociating DNA and a molecular weight of 6×10^6 ions was found in this solvent (Fig. 2). If DNA at a concentration of 0.04% or less was treated with 4 M urea and subsequently diluted with water to 1.0 M urea the molecular weight was found to be 3×10^6 (Fig 2), but, if the same experiment is done with more concentrated DNA solutions (> 0.08%) then a molecular weight of 6×10^6 is found on a subsequent dilution, proving that 4 M urea was not able to split DNA at these higher concentrations. No reliable data could be obtained for DNA solutions in between the two ranges of DNA concentration studied (Fig. 1) but this is not surprising since the situation in this intermediate range (0.05 to 0.08%) would be expected to be very complex.

The reason for this remarkable concentration dependance of the dissociation of the twin molecule can not be found in a mass action phenomenon since the urea is present in vast excess, there can be no question that the equilibrium is completely on the side of the dissociated molecule. An explanation may be found in Professor W. KUHN's suggestion[2] that length-wise association of the double molecules would prevent the rotational movement of the two threads relative to one another and thus make the relatively facile disentangling process envisaged by KUHN impossible. Electron micrographs of DNA (Fig 3) show that it is possible for these long molecules to aggregate in parallel and in fact under the conditions of drying on the supporting film a whole range of aggregates have been observed.

Complete contact of this kind in urea solution would completely inhibit the torsional motion necessary for the untwisting and clearly the chance of its happening would rapidly increase with increasing concentrations. In order
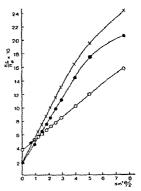
Fig. 1.—Limiting lines of Zimm plots of light scattering data of DNA from herring sperm (for definition of symbols see[1]). The intercept is inversely proportional to the weight average molecular weight of the dissolved DNA.

-●-●- 0.01% DNA containing 1.0 M urea and 0.2 M NaCl (Molecular weight 6×10^6).

-○-○- To 0.08% DNA an equal volume of 8 M urea was added (i.e. solution was 0.04% DNA in 4 M urea) and diluted 2 h later to finish up as 0.01% DNA containing 1.0 M urea and 0.2 M NaCl (Molecular weight ~ 3×10^6).

-×-×- To 0.16% DNA an equal volume of 8 M urea was added (i.e. solution was 0.08% DNA in 4 M urea) and diluted 2 h later to finish up as 0.01% DNA containing 1.0 M urea and 0.2 M NaCl (Molecular weight 6×10^6).

to prevent this aggregation during the drying of the specimen on the E. M. grid it is necessary to dilute the solution 100 fold from that used to prepare the specimen shown in Figure 3. So it is clear that there exists, despite the repulsive action of the ionized phosphate groups, strong attractive forces between DNA molecules. The failure to observe any aggregation by light scattering shows that this can only be of a very temporary nature although the small value of the second virial coefficient may be regarded as suggestive of interparticle attraction.

So far we have been unable to demonstrate a similar splitting by calf thymus nucleic acid as it is ordinarily prepared[3]. After prolonged exposure to a relatively concentrated solution of a sequestering agent, sodium ethylene-diamine tetracetate (versene), it was found that in 4 M urea this too slowly split into two half molecules of molecular weight 3×10^6. Since JUNGNER[4] had reported the presence of a surprisingly large amount of magnesium in calf thymus DNA it seemed possible that these bivalent ions might be responsible for the greater stability of DNA from this source. But we have been unable by trace metal analysis to confirm JUNGNER's findings and can detect no significant amounts of magnesium or any other trace metal in DNA obtained from

[1] P. ALEXANDER and K. A. STACEY, Biochem. J. **49**, 191 (1955).
[2] W. KUHN, Exper. **11**, 304 (1957).
[3] P. ALEXANDER and K. A. STACEY, Nature **176**, 162 (1954).
[4] G. JUNGNER, Science **113**, 378 (1951).

of a dose of γ-rays. Apart from Svedberg and Brohult, this technique has been used quantitatively by Sanigar and colleagues (4) and by Sheraga and Nims (9) in studies of the action of x-rays on fibrinogen solutions.

Our results bear a qualitative resemblance to those of Sheraga and Nims, though differing noticeably in detail. We have also observed an apparently exponential decrease of normally sedimenting material with increasing dose of radiation. We have also used appropriate compounds to protect the protein against the effects of radiation, and noted two seemingly distinct mechanisms of protections.

Experimental

Materials

The serum albumin used was obtained from A. B. Kabi, Stockholm. It was prepared by the Cohn method and, although electrophoretically pure, showed two components when examined in the ultracentrifuge. Of these two components, the overwhelmingly more abundant one was normal serum albumin; the second had a sedimentation constant slightly higher than that of the first, but insufficiently so to effect separation of the two. The two components thus always appeared under one peak in the sedimentation diagrams. With fresh material, about 80% of the dissolved protein appeared in the main peak of the sedimentation diagram of un-irradiated solutions.

Solutions for irradiation were made in water double-distilled in Pyrex glass. These solutions, rather than buffered ones, were used to avoid interference by salts with the radiation-chemical reactions. [cf. (6)]. Before use, the water was left standing overnight to bring it into equilibrium with the atmospheric oxygen.

Radiation

The solutions were irradiated with γ-rays from 6.3 curies (C.) of Co^{60} supplied by the Atomic Energy Research Establishment, Harwell, in the form of a hollow cylinder, inner diameter 2 4 cm, height 8.0 cm. Irradiations were made with the solutions in stoppered test tubes placed inside the cylinder. The dose rate was determined:

(a) *semi-empirically*, according to figures for a similar source given by Saunders, Morehead and Daniels (10) as 10,500 r./hr.

(b) *chemically*, by the degradation of polymethacrylic acid (PMA) according to the method of Alexander and Fox (11). The PMA was calibrated against a well-

(c) *physically*, by means of a thimble ionization chamber (12) calibrated against x-rays as above, and giving a result of 10,200 r./hr.

Considering the variety of errors in each method of calibration, the agreement is highly satisfactory, and the dose rate was taken as 10,250 r./hr.

Effects of Gamma Rays on Solutions of Human Serum Albumin. I. Sedimentation Studies.

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INTRODUCTION

Various studies have been made of the effects of ionizing radiations on proteins, and several reviews have been published (1, 2). Many of the studies have been of complex proteins with prosthetic enzyme groups, but some have been specifically concerned with serum albumin. Early investigators (3) noted changes in optical properties, viscosity, and pH after irradiation. Sanigar, Krejci, and Kraemer (4), working with human serum albumin, detected no change after irradiation with 29,000 r. of x-rays. Carrol, Mitchell, and Callanan (5), working with bovine serum albumin, detected "polymerization" of the protein after doses of up to 1,500,000 r. Barron and Finkelstein (6), also working with bovine serum albumin, and observing changes in ultraviolet absorption by the material after different doses of radiation, inferred an indirect (free-radical) mechanism of action of x-rays on proteins in solution.

The present investigation, continuing the earlier work of Svedberg and Brohult (7) and of Brohult (8) on the effect of high-energy radiations on certain proteins, concerns the sedimentation of human serum albumin after γ-irradiation. As measured in the oil-turbine ultracentrifuge, the amount of material sedimenting normally was used to measure the effect

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the control samples. This indicates that no large fragments are broken off the protein molecule but does not exclude the possibility of small fragments such as amino acid residues. However, precipitation of the protein and analysis of the supernatant liquid with ninhydrin showed no
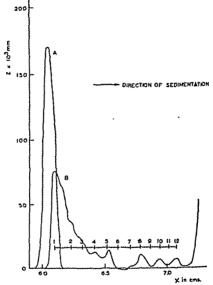
Fig. 1. Sedimentation diagrams by the Lamm scale method of (A) unirradiated and (B) irradiated solutions of 0.5% human serum albumin (dose: 91,000 r). Diagrams show sedimentation 20 min. after full speed was reached. Inset scale shows expected position of aggregates of 1, 2, 3, .. 12 protein molecules. Abscissa: radial distance in cm. Ordinate: scale line displacement in μ.

Measurements

Sedimentation measurements were made in one of the Uppsala Svedberg-type oil-turbine ultracentrifuges, using the Lamm scale method. Use is made of the Svedberg formula for the sedimentation, constant (13) namely,

$$s = \frac{MD(1 - Vp)}{RT} \tag{1}$$

where M is the molecular weight, D the diffusion constant, and V the partial specific volume of the solute; p is the density of the solution, R the gas constant, and T the absolute temperature For spherical molecules in particular, but generally for compact molecules of the same shape, s is proportional to $M^{2/3}$ if the other variables are kept constant

Experimental Methods

For each experiment a fresh stock of solution was prepared from the crystalline protein; for an experiment consisting of the measurement of several irradiated samples, a fresh sample was drawn from the stock for each irradiation

Irradiations with doses found necessary to produce measurable effects generally took, with the concentrations used (0 5-2% solutions), between 5 and 25 hr.; they were made at the ambient temperature (18-20°C.) and samples were irradiated 3 ml at a time. Between irradiation and measurement, irradiated solutions were kept together with the stock (control) solution at 4°C. Measurements were usually made within 48 hr of irradiation Solutions were untouched between irradiations and measurements

RESULTS

Effect of a Single Dose of γ-Rays

In Fig. 1 are shown the sedimentation diagrams, 20 min. after full speed (60,000 r.p.m. ≅ 250,000 × g) had been reached in the ultracentrifuge, of a 0.5 % solution of serum albumin (A) unirradiated (control) and (B) after a dose of 91,000 r. of Co⁶⁰ γ-rays. The most striking difference between the two is the presence of components of higher sedimentation constant that appear in the irradiated sample; this is taken as an indication of the aggregation of the protein molecules after irradiation. Of the amount of material present as shown in the diagram of the control, 92 % appears in the diagram of the irradiated sample. Nearly all the remaining 8 % appears in material which has already sedimented to the bottom of the cell, but which can be seen in earlier sedimentation diagrams.

No change in the region of sedimentation constant lower than that of the main peak has been found in the irradiated samples compared with

The results have been compared with the equation

$$C = C_0 e^{-\mu D/C_0} \qquad (2)$$

Values of μ, calculated by the method of least squares, are given in the last column of Table I, and determine the lines drawn in Fig. 2.

In solutions irradiated so that the amount of unaggregated material was less than 60% that of the control, a cloudiness was visible. In solutions irradiated so that the amount of unaggregated material was less than 40%, this cloudiness became a precipitate, progressively increasing with dose. To avoid possible interfering effects from the precipitate, doses of radiation were kept small enough for it not to appear.
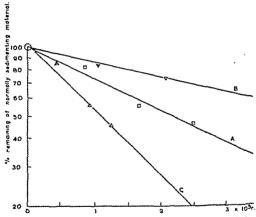
Fig. 2. Variation with dose of amount of material sedimenting normally for different initial protein concentrations. Curve (A) 1.0% protein solution; (B): 2.0%, (C) 0.5%. Abscissa. dose. Ordinate. logarithmic scale of percentage remaining of normally sedimenting material.

difference in amino acid content between irradiated and control solutions.

In Fig. 1 are shown the positions to which molecules would have sedimented if their sedimentation constants were $n^{2/3}s$, i.e., if their weights were nM and if they were of uniform shape [cf. Eq. (1)], for n up to 12, where s and M are the sedimentation constant and molecular weight, respectively, of the original serum albumin molecules. The undoubted correlation between some of these positions and the peaks in the diagram of the irradiated solution reinforces the suggestion of simple aggregation of the protein molecules as a result of irradiation. This interpretation, however, cannot be strictly maintained without further separation of the products of the irradiation.

Variation of Effect with Dose

The variable chosen for comparison was C, the concentration of unaggregated protein molecules as measured in the sedimentation diagram 80 min. after full speed had been reached. This concentration was determined from the area under the peak after appropriate corrections (13). In a given experiment, the concentration expressed as a percentage of C_0, that of the control, has been considered as a function of the dose, D. The results of a group of experiments are given in Table I and shown in a semilogarithmic plot in Fig. 2.

TABLE I

Effect of Radiation on Amount and Sedimentation Constant of Unaggregated Protein Material

C_0, per cent concentration	D, dose	$100\,C/C_0$, unaggregated protein	Sedimentation constant, S	Average sedimentation constant, S	$\mu \times 10^8$
	r	*% of control*			
	0	100	4 43		
0 5	42,000	85	4 13		
	90,700	56	5 16	4 59	332
	123,000	46	4 66		
	0	100	3 99		
1.0	82,400	82	4 29	4 23	326
	164,000	56	4.28		
	246,000	47	4 35		
	0	100	3.81		
2.0	103,000	83	3.90	3 92	306
	206,000	73	4 04		

Sodium benzoate also acts as a partial protector against the effects of radiation (Fig. 4). In this case, however, for a given concentration of sodium benzoate with respect to serum albumin, the amount of unaggregated protein material decreases exponentially with increasing dose, i.e., as $e^{-\mu'D}$. The exponential index, μ', itself decreases with increasing relative concentration of sodium benzoate. On the other hand, we have found that sodium acetate used in the same way as the sodium
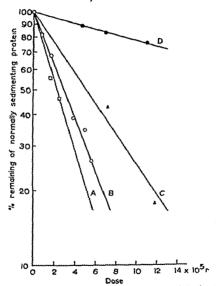
Fig. 4 Protection against radiation of protein in 10% solution by sodium benzoate. Curve (A): no benzoate; (B) 4 × 10⁻⁴ benzoate; (C) 1 × 10⁻³ M benzoate; (D) 7 × 10⁻³ M benzoate

Action of Protective Agents

A study has been made of the protective effects of four substances: thiourea, β-mercaptoethylamine, sodium benzoate, and sodium acetate. Results from the first two are shown in Fig. 3. It was found that a concentration of 10^{-3} M of thiourea in a 1 % solution of serum albumin appeared to protect the protein molecule completely, with respect to the particular changes studied, against the first 140,000 r. (approx.). For higher doses the amount of unchanged protein material decreased as in the case with no protection. Twice the concentration of thiourea gave complete protection against twice the dose of radiation. β-Mercaptoethylamine acts similarly, and the protein remains partly protected after the initial complete protection has ceased.
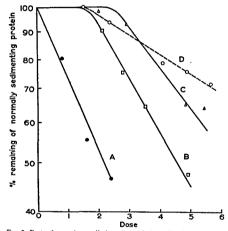
Fig. 3. Protection against radiation of protein in 1 0% solution, by thiourea and β-mercaptoethylamine. Curve (A): no protecting agent; (B) 1 × 10^{-3} M thiourea; (C) 2 × 10^{-3} M thiourea (D) 2 × 10^{-3} M β-mercaptoethylamine.

According to Eq. (2), which is that given by Dale and colleagues (15), the exponential index μ should be independent of the initial protein concentration C_0. However, from Table I μ is seen to decrease slowly with increasing C_0. But this decrease is of the order of the experimental error and is probably fortuitous.

Extrapolating from the results given in Table I, it is found that for a 1 % solution the amount of unaffected material is reduced to 37 % by a dose of 310,000 r. Taking the molecular weight of the protein as 67,000 (13) and the number of ion-pairs produced as 1.79×10^{12}/r./ml. (16), this dose produces 6.2 ion-pairs per molecule. If a single free radical (the product of a single ion-pair) is sufficient to affect a single protein molecule, it follows (16) that the ionic efficiency is 0.162 (or $G = 0.49$).

Protective Agents

A concentration of 10^{-3} M thiourea gives apparently complete protection to a 1 % solution of serum albumin for doses up to 140,000 r., and doubling the thiourea doubles the threshold of dose at which the protein begins to be affected. This suggests that thiourea protects with very great efficiency while it exists in solution, but that is is transformed to an inert material by the free radicals produced by the radiation, with an ionic efficiency of 2.6 ($G = 7.9$). This figure is in agreement with that given by Dale and Davies (18) for much higher concentrations of thiourea. The protection afforded by sodium benzoate suggests that the competition for free radicals by the benzoate is relatively weak, but that the products of the benzoate and free radicals continue to interact successively with the radicals, and with approximately constant G-values. While a succession of interactions of sodium benzoate with free radicals is known (18), the G-values involved do not appear to have been determined. The contrast between the protection by sodium benzoate and by thiourea is notable. Protection by β-mercaptoethylamine is intermediate.

benzoate, with the same pK and pH values, and at the same concentration, gave no protection whatsoever.

DISCUSSION

Sedimentation Constant

Since the measurements were made on unbuffered solutions they were not expected to give good values of the sedimentation constant. However, the average value for the unirradiated solution, 4.07 S, is in good agreement with the value obtained by Charlwood (14). Charlwood also showed that the sedimentation constant varied with the method of preparation of the protein material, with the composition of the buffer, and with the protein concentration. The sedimentation constants obtained in the present experiments are given in Table I. The average for each concentration varies with the concentration, in agreement with Charlwood's results, and the agreement extends (for most of the present results) to the variation with the amount of normally sedimenting material after irradiation of a solution of given concentration. Some small changes in sedimentation constant were noted by Sanigar and colleagues (4) after ultraviolet irradiation. The conclusion drawn here, as also by those authors, is that the sedimentation constant of unaggregated material remains essentially unchanged.

Variation with Dose of Amount of Material Sedimenting Normally

Within experimental error, the amount of material sedimenting normally decreases exponentially with dose. The variation of magnitude of effect with protein concentration following a given dose of radiation removes an essential condition for a direct mechanism of action of the radiation on the protein molecules. For this reason it must be assumed, as was done by Barron and Finkelstein (6), that the effect is indirect and is due to the actions of free radicals produced in the solvent by the radiations.

An exponential decrease of enzymic activity with x-ray dose was noted by Dale, Meredith, and Tweedie (15) in the irradiation of carboxypeptidase, and interpreted by these authors as indicating continued competition for free radicals by affected enzymes. Although our system and method of measurement is quite different, our results suggest, similarly, that each protein molecule is able to interact with more than one free radical, so that affected molecules can compete with unaffected ones for the radicals.

Effects of Gamma Rays on Solutions of Human Serum Albumin. II. Chromatographic Studies

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INTRODUCTION

As a further application of a recent procedure for protein chromatography on the anion-exchange resin Dowex 2 (1), a study was made of the effect of irradiation with γ-rays on the chromatographic behavior of human serum albumin. This work also served to supplement an investigation of the sedimentation behavior of the irradiated protein (2). Changes in irradiated proteins have been studied by several physical and chemical methods, but this seems to be the first use of chromatography for the purpose.

MATERIALS AND METHODS

Chromatographic Technique

The experimental details for chromatography of proteins on Dowex 2 have recently been described (1). THAM-HCl buffers have been used for elution, with the pH kept constant at 7.35. All molarities cited below refer to the concentration of THAM The dimensions of the column were 18 × 1.5 cm., and the same column was used in all experiments. The fraction collector here used worked on a time basis and fractions were shifted every 30 min., giving fraction volumes of about 3 5 ml. All chromatographic experiments were run in a cold room at 4°

Serum Albumin

The sample used was provided by A. B. Kabi, Stockholm, and was prepared by the Cohn method. It was examined in the ultracentrifuge, where about 80% of the material applied sedimented as a symmetric peak with the sedimentation constant of native human serum albumin About 80% of the material applied to

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SUMMARY

1. The oil-turbine ultracentrifuge has been used to measure quantitatively an effect of γ-rays on human serum albumin in salt-free solution. The effect is shown to be due to the indirect action of the radiation.

2. The amount of protein with normal sedimentation constant decreases exponentially with dose. The remainder appears as a series of aggregates

3. Thiourea and sodium benzoate are both found to protect the protein against radiation, but apparently in different ways.

REFERENCES

1. BACQ, Z. M, AND ALEXANDER, P., "Fundamentals of Radiobiology," Butterworths, London, 1955
2. HOLLAENDER, A, ed., "Radiation Biology", McGraw-Hill, New York, 1954.
3 ARNOW, L. E., *Physiol Revs*, **16**, 671 (1936)
4 SANIGAR, E. B, KREJCI, L E, AND KRAEMER, E. O., *Biochem. J.* **33**, 1 (1939).
5 CARROL, W. R, MITCHELL, E R, AND CALLANAN, M. J., *Arch Biochem. Biophys* **39**, 232 (1952).
6 BARRON, E. S. G, AND FINKELSTEIN, P., *Arch. Biochem. Biophys* **41**, 212 (1952).
7 SVEDBERG, T, AND BROHULT, S, *Nature* **142**, 830 (1938); *Nature* **143**, 938 (1939)
8. BROHULT, S., *Nova Acta Regiae Soc. Sci. Upsaliensis* **12**, No. 4 (1940).
9 SHERAGA, H A., AND NIMS, L. F., *Arch Biochem. Biophys* **36**, 336 (1952).
10 SAUNDERS, D F., MOREHEAD, F. F., AND DANIELS, F, *J. Am Chem. Soc.* **75**, 3096 (1953)
11 ALEXANDER, P., AND FOX, M., *Trans. Faraday Soc* **50**, 605 (1954).
12 TURNER, R C, AND NEWBERY, S P, *Brit J Radiol.* **23**, 424 (1950).
13. SVEDBERG, T., AND PEDERSEN, K O, "The Ultracentrifuge," Oxford Univ. Press, Oxford, 1940
14 CHARLWOOD, P. A., *Biochem J* **56**, 259 (1954)
15 DALE, W. M, MEREDITH, W. J., AND TWEDIE, M C K., *Nature* **151**, 280 (1943)
16 LEA, D E, "Actions of Radiations on Living Cells," Cambridge Univ. Press, Cambridge, 1947.
17 DALE, W M., AND DAVIES, J V, "Progress in Radiobiology", Oliver and Boyd, Edinburgh, 1956.
18 LOEBL, H, STEIN, G., AND WEISS, J, *J Chem Soc* **1951**, 405

tions 11–16 were ignored. For the experiment shown in Fig. 1, curve *A*, the recovery so obtained was 79%.

For a 1% solution irradiated with 221,000 r. (Fig. 1, curve *B*), the amount of material eluted with the 0.25 *M* buffer was reduced to 43% while the amount of material eluted with 1 *N* HCl was increased. The material in the first peak was examined in the ultracentrifuge after concentration by pervaporation. It was found to give a single symmetrical peak in the sedimentation diagram, with sedimentation constant 4.23 *S*, (1% solution), the same as that found for unchromatographed material. Also the ultraviolet extinction between 240 and 300 mμ was that to be expected for pure serum albumin.

The material eluted by the acid was found to be so severely denatured that the extinction maximum at 279 mμ had disappeared (and with it the minimum at 250 mμ). The denaturation was found to be much greater than that resulting when either unirradiated or irradiated protein ma-
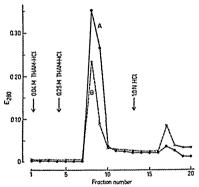
Fig 1. Chromatographic experiments with 10 mg. of unirradiated (curve *A*) and irradiated (curve *B*) human serum albumin on the same column (18 × 1.5 cm) of Dowex 2. The first zone is eluted with 0 25 *M* THAM–HCl buffer, pH 7 35; the second zone is eluted with 1 0 *N* HCl which also regenerates the column The dose of radiation was 221,000 r

the chromatographic column was eluted with a buffer concentration of 0.25 M, this being in close agreement with minimum strength of buffer required for the complete elution of albumin from a normal human serum (1). It is thus clear that the albumin sample behaved chromatographically as the protein in the native state and did not show the abnormality found earlier for another commercial sample (4)

Radiation

The material was irradiated with γ-rays from 6 3 curies of cobalt-60. The cobalt, supplied by Atomic Energy Research Establishment, Harwell, was in the form of a hollow cylinder of length 8 cm and internal diameter 2 2 cm The dose rate within this cylinder was found to be 10,250 r./hr

Experimental Methods

Each experiment was carried out in the following manner a fresh solution was prepared by dissolving the crystalline protein material in double-distilled water, separate samples of 2-3 ml were then irradiated as required in test tubes placed within the cylinder of cobalt. Between irradiation and chromatographic examination the samples were left with the control at 4° The examinations were all made within 72 hr of irradiation.

Immediately before the start of the chromatographic experiment the sample to be examined was diluted 1:1 with 0.08 M buffer, and a measured amount of the resulting solution was applied to the column, equilibrated with 0 01 M buffer The maximum amount of protein material applied was 1 ml. of a 1% solution. When the albumin sample had sunk into the resin, elution was first carried out with 0.04 M buffer. After 2 hr a stepwise change was made to elution with 0 25 M buffer. After a further 3-4 hr. a second stepwise change was made to elution with 1 N HCl. About 3 hr. later the experiment was stopped

The amount of protein in the different fractions was followed by measuring the extinction at 280 mμ using a Beckman DU spectrophotometer and 1-cm cells Distilled water was always used as a blank

RESULTS

Effect of a Single Dose of γ-Rays

When an unirradiated (control) solution of albumin is chromatographed (Fig. 1, curve A) most of the material applied to the column is eluted with 0.25 M buffer while only a small amount is eluted with 1 N HCl. As the tris(hydroxymethyl)aminomethane (THAM)-HCl buffers always contain some ultraviolet-absorbing material (the extinction of a 0 25 M solution is 0 055), it is difficult to judge to what extent the tailing of the peaks is due to protein material. For this reason the recoveries were calculated only from the extinction of fractions 8-10, while frac-

ponentially with dose, that of the second increases linearly. The curved line in Fig. 2 is drawn through the values expected from the previous sedimentation studies (2).

DISCUSSION

It is generally accepted that the effects of ionizing radiations on proteins in solution are caused by free radicals produced in the solvent(3). It *is probable that these radicals will change the surface properties of the protein molecules and hence change their chromatographic behavior.*

It is striking that the relation between unaffected material and dose of radiation is almost the same whether obtained by chromatography or sedimentation studies (see Fig. 2). The closeness of the two sets of results suggests that the same effect is measured in each case. *This idea is supported by the fact that that part of irradiated serum albumin with the chromatographic properties of native material also sediments normally.*

Because of the denaturation of the material in the second zone, eluted with hydrochloric acid, the ultraviolet extinction is not, in this case, a measure of the amount of protein eluted. It has also been found that, with the present size of column, the resin is poisoned (i.e., ceases to effect chromatographic separation) after about 12 experiments with protein irradiated with average doses. Since such poisoning is not observed after more than twice this number of other experiments (1) with unirradiated material, it appears that some of the protein affected by radiation is absorbed irreversibly on the resin. Thus while the curved line in Fig. 2 gives a genuine reflection of the decrease of the amount of unaffected protein, the straight line does not indicate the amount of denaturated protein. We are unable at present to give any interpretation of the apparent linearity in the relation between dose and the added extinctions of the second zone.

It is evident that the type of chromatography on Dowex 2 used here successfully separates native from aggregated protein molecules. It thus provides a rapid and convenient, as well as cheap method of studying an effect of ionizing radiations on proteins.

ACKNOWLEDGMENTS

terial was left standing in 1 N HCl for the same time as in a chromato-
graphic experiment. For this reason it was not possible to check that,
for the irradiated sample, the material lost from the first peak did ap-
pear in the second.

Variation of Effect with Dose

The totals of the extinctions of the fractions comprising the first and
second peaks have been considered separately as functions of dose.
Figure 2 shows the relationship between these variables for one series of
experiments. While the total extinction of the first peak decreases ex-
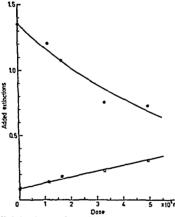
Fig. 2. Variations between dose and the added extinctions of the different
fractions comprising the two zones obtained in chromatographic experiments like
those shown in Fig 1. O, 1st zone; material eluted with 0.25 M THAM-HCl buffer.
The curved line is drawn through values expected from previous sedimentation
studies. □ 2nd zone; material eluted with 1 N HCl. The straight line is drawn
through the experimental points.

Fibre formation by cellular systems

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[Plates 2 to 6]

In order to study possible mechanisms of fibrogenesis within living cells, experiments have been carried out using solutions of nucleo-proteins to determine the conditions which lead to the building up of spontaneously ordered structures. Nucleo-protein solutions which separate into a two-phase system produce orientated fibres by a process of aggregation. These fibres are birefringent and show visual dichroism after staining with vital stains. A method of measuring concentrations by interference microscopy in the living cell has shown that the concentration of high molecular-weight material within the nucleus would be expected to maintain ordered structures of the type observed in the solutions of isolated nucleo-protein. It is shown that the formation of fibres takes place by a similar process in the cytoplasm during spermatogenesis. These fibres also undergo a process of spiralization which is analogous to the behaviour of chromosomes.

INTRODUCTION

The steps involved in the synthesis of biological macromolecules have been the subject of considerable interest in recent years. But from many points of view, the later stages, which lead to the formation of larger structures, are equally important. Fibrogenesis has been studied in extracellular systems such as silk and in considerable detail in the case of collagen (Jackson 1956). But apart from the development of the mitotic spindle, little is known about such processes in the case of nuclear and cytoplasmic organelles.

In earlier papers, evidence for a fibrous structure of the chromosomes has been described (Ambrose & Gopal-Ayengar 1952, 1953; Ambrose, Cuckow & Gopal-Ayengar 1954). Physico-chemical studies of the behaviour of the chromosomes within the fresh nucleus have indicated the presence of anisotropic structures suggesting the presence of fibrils running parallel to the length of the chromosome. In addition, electron microscopy has shown the presence of microfibrils of 100 to 200 Å diameter running parallel to this direction. Similar results have been obtained by Yasuzumi, Odate & Ota (1951). Physico-chemical studies have also suggested that the microfibrils might be formed by the linear aggregation of small particles, through secondary forces of cohesion. This view is suggested by indications of a beaded structure at intervals of 700 Å along the length of the microfibrils seen in the electron-micrographs. Recent studies have suggested that elements of small dimensions may in fact provide the elementary units of heredity. Evidence for a particulate structure of the chromosomes has also been given by Mazia (1954).

The present paper is in two parts; in the first part an attempt has been made to study certain colloidal properties of nucleo-protein solutions directly, so avoiding

SUMMARY

It has been demonstrated that the effect of γ-rays on human serum albumin can be followed by chromatography on Dowex 2.

REFERENCES

1. BOMAN, H G., AND WESTLUND, L., *Arch. Biochem. and Biophys.* 64, 217 (1956).
2. ROSEN, D., BROHULT, S., AND ALEXANDER, P., *Arch. Biochem and Biophys* 70, 266 (1957).
3. BACQ, Z M., AND ALEXANDER, P., "Fundamentals of Radiobiology," Butterworths, London, 1955.
4. BOMAN, H G., *Nature* 175, 898 (1955).
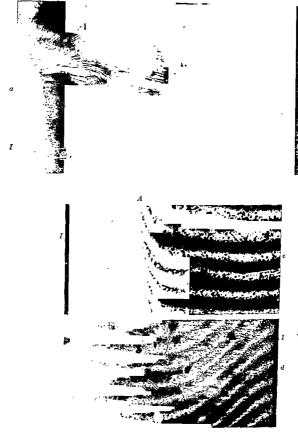
FIGURE 2 (a) Polarized light photograph of fibrous structures in calf thymus nucleo-protein.
(b) Polarized light photograph of diffuse region of birefringence in nucleic acid. ×160.
(c) Interferometer photograph showing diffusion gradients in calf thymus nucleo-protein.
×36. *I*, drying edge; *A*, region of sudden change in diffusion coefficient. (d) Interferometer photograph showing diffusion gradient in calf thymus nucleic acid with absence of sharp boundary. ×36.

artifacts which must inevitably arise in specimens fixed for electron microscopy. In the second part the fibre-forming properties of these solutions have been compared with the properties of the nucleus in a locust, by direct observations of living cells using interference microscopy.

EXPERIMENTAL METHODS AND RESULTS

(a) *The formation of spontaneously ordered structures in solutions of nucleo-protein and nucleic acid*

(i) *Concentration effects*

A solution of nucleic acid or nucleo-protein is allowed to concentrate slowly in the space formed between a microscope slide and coverslip, arranged in the form of a wedge. The slide is mounted on the stage of the polarizing microscope with crossed polarizers. As the concentration of the solution increases along the drying edge, spontaneously birefringent regions begin to appear in the solution. In plate 2*a* is shown a solution of calf thymus nucleo-protein. The solution of 1 % initial concentration was freshly prepared according to the method described by Shooter, Davison & Butler (1954). It was dialyzed against distilled water to remove salt. The process of concentrating the solution on the slide did not, therefore, lead to changes of salt concentration. In the region adjacent to the drying edge of plate 2*a*, the solution is strongly birefringent, but in addition many long fibrils can be seen penetrating into the dilute solution. The sign of the birefringence of these fine fibrils was found to be negative (maximum refractive index with the E-vector $\perp$ to the fibre axis). The more densely packed material was also negatively birefringent when referred to the drying edge of the cell.

A very low concentration of the dye, neutral red, was in some experiments introduced into the original solution. The fibrils which formed when the solution was allowed to concentrate were found to be stained. When viewed in polarized light (polarizer alone) they showed marked dichroism, with a maximum absorption when the E-vector was perpendicular to the axis of the fibril.

Similar experiments were carried out using solutions of calf-thymus nucleic acid. This material was supplied by Professor Butler and Dr Davison. A 1 % solution in distilled water was allowed to concentrate as before. In this case the birefringent region formed along the drying edge of the slide, but the boundary of this region was diffuse; no visible fibrils could be seen in any instance within the solution. The sign of the birefringent region was again negative when referred to the drying edge of the cell.

In order to relate the self-orientation properties of the solutions more exactly to the concentration changes, an interferometer was used to measure the concentration-distance curve. A cell was prepared by cementing two microscope coverslips (no. 3) on to two narrow strips of microscope slide. A drop of nucleo-protein solution was introduced into the channel; one surface of the drop was held in contact with the end of the channel by surface tension. The other surface was in the interior of the cell. Evaporation of water from the surface took place until a concentration gradient was set up in the channel, as in the previous experiments.
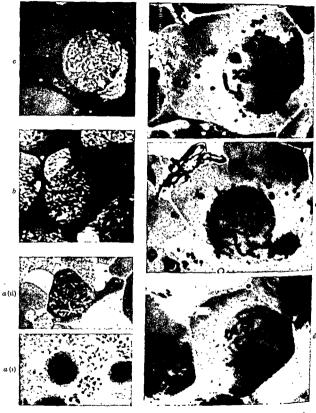
FIGURE 4. Stages of meiosis in *Schistocerca gregaria*. (a) Spermatogonium in unflattened and flattened condition showing dark threads in nucleus (positive contrast). ×960 and ×830. (b) Young spermatocyte showing light threads in nucleus (negative contrast). ×860. (c) Leptotene stage (negative contrast). ×980. (d) Bouquet stage (slightly hypertonic medium). ×1120. (e) Pachytene stage (slightly hypertonic medium). ×720. (f) Early diakinesis (slightly hypertonic medium). ×1210.
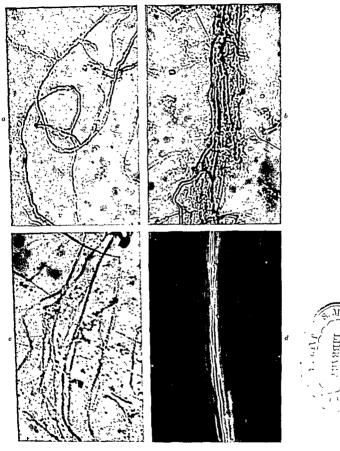
FIGURE 3 (a, b) Fibres formed from T_2 phage particles. Dried on slide. × 1000. (c) Phase-contrast photograph of fresh fibres. × 1600. (d) Polarized light photographs of a bundle of fresh fibres. × 160.

Ambrose

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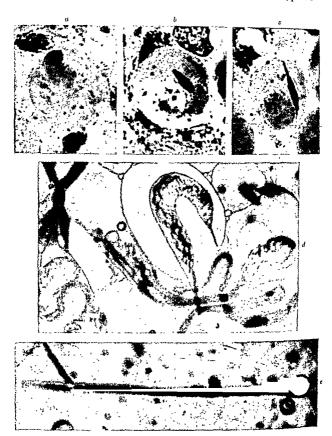
FIGURE 6. (*a–c*). Stages in elongation of tactoid. ×1300. (*d*) Tactoid flattened at stage 9 and showing interwoven helical fibres. ×2500. (*e*) Mature sperm (negative contrast) showing nucleus (white), tail element (light) and surrounding sheath cytoplasm (dark). ×1300.
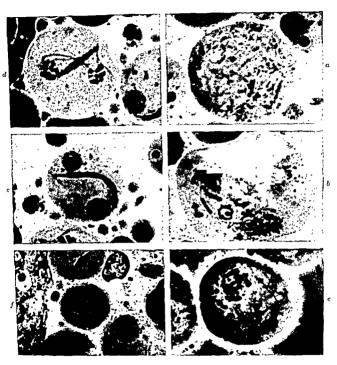
FIGURE 5. Stages of meiosis. (a) Pachytene showing particles in cytoplasm. × 740. (b) Diakinesis stage showing short fibrils in cytoplasm. × 680. (c) Secondary spermatocyte showing very long and thin fibres in cytoplasm. × 1230. (d) Cell flattened during last meiotic division showing bundle of fibres at telophase. × 760. (e) The same, very late telophase, showing *transverse* division of fibrous bundle. × 820. (f). Young spermatid, showing fibrous bundle aggregated into dense sphere attached to nucleus. × 850.

addition to the virus particles; for example, free deoxyribosenucleic acid which is produced in excess in some cases during synthesis of the virus. But it was found that solutions of free nucleic acid, treated under the same conditions produced completely unorientated precipitates, indicating that the fibre-forming properties are mainly dependent on the behaviour of the virus particles.

The fibres are birefringent (figure 3d, plate 3) and show considerable dichroism on staining with toluidine blue or neutral red. (Of the same sign as the calf thymus nucleo-protein.) They are also elastic and can be stretched reversibly with a micro-manipulator to over twice their original length. They require the presence of water for the maintenance of their organization because the dichroism and birefringence are lost on drying.

(b) The concentration of high molecular weight material within the living cell

Following the studies of the model systems, it was considered that an examination of the concentration of high-molecular-weight material within the nucleus of the living cell, would be of value in interpreting the behaviour of this complex colloidal system. For the study of small objects such as living cells, the divided beam type of interference microscope more recently developed by Dyson (1949) and by Smith (Brit. Pat. 639014) is to be preferred to the multiple beam system already described. Davies & Wilkins (1951) and Barer (1952) have used the interference microscope to determine the mass of high-molecular-weight material in living cells, with much success (see also Mellors, Kupfer & Hollander 1953).

The specific increments (increase of refractive index for addition of 1 g to 100 ml. of solution) are very similar for many proteins (Adair & Robinson 1930) and for nucleic acid; Barer & Tkaczyk (1954) conclude that the refractive index provides an accurate measure of the mass per unit volume of high-molecular-weight material within the cell; the contributions from salt solutions and other low molecular weight materials are very small.

If the mass per unit area had been determined in a biological structure, a measurement of the cell thickness would enable the concentration to be determined. With spherical cells, the thickness is known from the geometry, but in most specimens seen on the microscope, the thickness is not known. The following simple technique has been worked out to enable the concentration to be measured for those living cells which can be suspended in their natural medium.

(i) A drop of cell suspension is mounted on a slide and covered immediately with a coverslip; the volume of the drop should be insufficient to fill the whole area of the coverslip after it has been drawn out by capillary attraction. No mechanical pressure should be applied. The edges of the coverslip are sealed by painting with a molten mixture of paraffin wax and Vaseline. Care should be taken to avoid the presence of dust particles on the surfaces. By varying the volume of the drop it is possible to control the thickness of the liquid layer with reasonable accuracy in the range from 5 to $10\,\mu$. In this layer, the cells are compressed between the surface of the slide and coverslip. If the cell is not compressed to less than half or one-third of its normal diameter and is adjacent to an air bubble on the slide, to

The birefringent regions could be examined in the polarizing microscope, while the concentration gradient was examined with an interference microscope. This particular interferometer has been described previously (Ambrose 1948). A pair of partially silvered glass plates produce multiple beam interference fringes when illuminated by an adjustable collimating system using monochromatic light. This microscope has been used by Robinson (1950) for the study of diffusion gradients in polymer films. Multiple beam interference fringes provide an extremely sensitive method of detecting small changes of path difference over a given area, as has been shown in the case of surface contours in the well-known experiments of Tolansky (reviewed by Tolansky (1948)).

The interference microscope has recently been modified (Easty & Ambrose 1957) so that the silvered surfaces are completely protected and permanent. The small cell may be readily inserted and withdrawn from the instrument, without disturbing the silvered surfaces.

To study the concentration gradient in the nucleo-protein solution, the fringe system is adjusted to run parallel to the length of the channel. An increase in concentration causes a vertical and upward displacement of the fringes to occur. In figure 2c, plate 2, is shown a curve obtained with the nucleo-protein solution already described. In figure 2d, plate 2, is shown a similar curve obtained with the nucleic acid solution. The refractive index and concentration of protein show a linear relationship up to quite high concentrations (Barer & Tkaczyk 1954). The fringe system therefore provides a representation of the concentration-distance curve along the channel.

For nucleic acid, the curve obtained is of the smooth type most usually encountered with diffusion processes. But with the nucleo-protein, a sharp change of gradient occurs at the point I. This change occurs in the range of 1 to 3 % concentration of nucleo-protein. A change of this type indicates that a sharp change in the diffusion coefficient has occurred. It can be correlated with a sudden increase in the degree of association of the macromolecules at a critical concentration (Robinson 1950). An aggregation process is taking place in the solution of nucleo-protein, but no such process is indicated with nucleic acid.

(ii) *The formation of orientated fibres by precipitation*

Birefringent and dichroic fibres can be obtained from solutions of biological material by careful precipitation in some instances. At neutral pH, a solution of T_2 bacteriophage-virus, obtained by infection of a bacterial culture, shows Tyndall scattering. This is due to the presence of virus particles of 800 Å diameter with tails of length 1000 Å (Avery, MacLeod & McCarty 1944). On reducing the pH of the medium to 4 by the slow addition of acid in the presence of phosphate buffer, extremely long fibres may form from the solution. At neutral pH the solution has a viscosity little higher than that of water, but a temporary increase in viscosity occurs before precipitation, suggesting that anisotropic aggregates are developing before the appearance of the visible fibres.

In solutions it is difficult to establish that other cellular constituents may not be involved in providing adhesive material for the formation of the fibres, in

If the refractive index of the medium is 1·3333 and the thickness of the liquid layer is t

$$\lambda \left(n + \frac{x}{180} \right) = t(1\cdot3333 - 1),$$

$$t = \frac{\lambda(n + x/180)}{0\cdot3333}$$

This method enables the thickness of the layer of medium to be determined to 1 part in 500 for thicknesses of 5 to 10μ.

(iii) The measuring beam is now arranged to pass through the flattened cell. If the retardation through the clear part of the cytoplasm is given by x_c

$$\frac{x_c}{180} = \frac{t}{\lambda} (\mu_c - 1\cdot3333).$$

The refractive index (μ_c) of the cytoplasm may be determined, because t, the thickness of the cell, is equal to that of the liquid layer (figure 1c).

The concentration of high-molecular-weight material is then given by $\frac{\mu_c - 1\cdot3333}{0\cdot00185}$,

where 0·00185 (α) is the value of the specific increment. (Increase of refractive index for addition of 1 g to 100 ml.)

The concentrations in the nuclear sap and in the nuclear inclusions may be similarly determined. In the measurements on nuclear inclusions, the initial diameter of the inclusion must always be greater than the thickness to which the cell is finally compressed; measurements made in the centre of the inclusion will then give a measure of the true concentration. For this reason the large chromosomes of Locusta are particularly suitable for these measurements. Corrections should strictly be made for the presence of cell membrane and nuclear membrane, but these are small compared with the total thickness of the cell and have been neglected in the measurements described below. If the refractive index of the medium differs appreciably from that of water (1·3333), the value may be determined on an Abbé refractometer.

(c) *Results obtained with living cells at various stages of meiosis in Schistocerca gregaria*

Cells observed at various stages of spermatogenesis in *Schistocerca gregaria* provide a particularly suitable system for the type of measurement described above. The cells may be separated from the tubules of the testis and the stage of meiosis may be recognized readily from the appearance of the nucleus as seen in the interference microscope. Photographs of living cells at various stages are shown in figure 4, plate 4.

Concentration measurements were made on the clear portion of the cytoplasm, nuclear sap and chromosome material in groups of cells at stages from spermatogonium to development of the mature sperm, prepared in the following manner.

The testes were isolated in a drop of amphibian Ringer solution. A single tubule was then mounted by the method already described. The surface tension was

provide an adequate supply of oxygen, it will remain healthy under such conditions for a considerable time (up to 24 h with *Ascites* tumour cells (Easty, Ledoux & Ambrose 1956)).

(ii) In the Smith (Baker) shearing system of interference microscopy, effectively two beams are used, as shown in figure 1. The reference beam R is allowed to pass through the mounting medium of the cell, while the measuring beam M passes through the air bubble. The analyzer is rotated in *monochromatic light* until the medium appears dark; it is then set for the air bubble to appear dark. If the
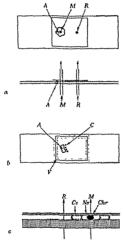
FIGURE 1. Method of measuring concentrations in living cells. A, air bubble; R, reference beam; M, measuring beam; C, cells adjacent to air bubbles; Cs, cytoplasm; Ns, nuclear sap; Chr, chromosome; V, Vaseline.

difference between the two readings is $x°$, $x/180$ gives the fractional number of wavelengths retardation. The air bubble will generally show three to four whole wavelengths retardation. The number of whole wavelengths retardation is determined in white light, using a fringe eyepiece. The displacement of the fringes in the air bubble compared with the medium is observed, and their number n determined for the black fringe of zero order. The total retardation through the air bubble relative to the medium is then equal to

$$n\lambda + \frac{x\lambda}{180}.$$

(iii) This material is carried over during the first meiotic division and in the cytoplasm of the interphase cell it builds up very long and straight fibres which may stretch right across the cell diameter (figure 5c).

(iv) During the second meiotic division the fibrils are lined up parallel to the spindle in the form of a bundle and are drawn out at telophase and split transversely (figures 5d, e).

(v) The mass of material condenses in each spermatid into a sphere of high concentration which remains attached to the nuclear membrane (figure 5f).

(vi) The sphere slowly elongates into a tactoid shape (figures 6a, b, c, plate 6).

If examined at this stage under high power after flattening in the fresh condition, it can be seen that the organelle is composed of a fine network of *helically* interwoven fibres (figure 6d).

DISCUSSION

The results described in the experimental section indicate that spontaneously ordered structures form quite readily in solutions of calf thymus nucleo-protein and T_2 bacteriophage. The building of such structures has been described previously by Bernal & Fankuchen (1937) in experiments with tobacco mosaic virus and in solution of synthetic polypeptides by Elliott & Ambrose (1950). In both these systems moderately large regions of birefringence were observed; they occurred in tobacco mosaic virus as the spindle-shaped structures known as tactoids, and in the synthetic polypeptides as the spherical bodies with a tangential arrangement of elongated molecules which are known as spherulites. The solutions of calf thymus and bacteriophage nucleo-protein are remarkable in that they form birefringent fibres.

The sharp change in the diffusion coefficient which takes place in the concentration range of 1 to 3% in the thymus material suggests that the fibres are produced by an aggregation process (figure 2c, plate 2).

In the virus solutions aggregation of particles almost certainly takes place. Structures of this kind may be likened to the liquid crystals which form in solutions of certain types of small molecule, they are intermediate in organization between true crystals and solutions, a high proportion of liquid medium being present in the organized phase. The physico-chemical conditions which favour their formation are rather similar to those which lead to the formation of solid crystals, i.e. a slow increase of concentration under very still conditions, or an exactly controlled reduction of solubility. Such processes differ from those commonly employed for the preparation of synthetic fibres, where mechanical work must be done in order to produce orientation. In fibres drawn from the melt or from a volatile solvent the streaming process leads to orientation. Stretching or rolling of the solid fibre may also be used in some cases. But in biological systems the rates of flow are very small and fibres are much more likely to be produced by a process of self orientation, as described in the present paper.

The measurements which have now been carried out on the living cells of *S. gregaria* indicate that the concentration of high-molecular-weight material in the nuclear threads is at all stages much higher than the minimum concentration

sufficient to drive the cells out into the surrounding medium. Those cells, which were adjacent to an air bubble and were flattened to the required extent, were then examined.

TABLE 1. CONCENTRATIONS OF HIGH-MOLECULAR-WEIGHT MATERIAL MEASURED IN LIVING CELLS DURING MEIOSIS IN *SCHISTOCERCA GREGARIA*

(mean values within 10%)

| | nucleus | | |
	chromosomes (%)	nuclear sap (%)	cytoplasm (%)
spermatogonium			
resting	18	7	11
dividing	25–32	12	—
primary spermatocyte			
resting	18	6	9
leptotene	13	1	8
diakinesis	20	11	12
	whole nucleus		
secondary spermatocyte			
interphase	22	—	11
dividing	23–32	—	13
spermatids			
early	11	—	10
developing tail	11	—	6
mature sperm	23	—	14

Some results obtained are summarized in table 1. From these observations, it is clear that

(i) throughout the stages leading to the last mitotic division, first meiotic division and second meiotic division, the chromosomes are present as a distinct phase within the nucleus. The concentration of this phase is always high. It is always higher than the minimum concentration at which marked aggregation was observed in the solutions of nucleo-protein.

(ii) the concentration of the chromosome phase remains high during interphase between first and second meiotic division

(iii) the nuclear concentration increases during the development of the sperm head.

(d) Cytoplasmic fibres formed during meiosis

During the course of this investigation a particularly interesting example of fibrogenesis was observed in the cytoplasm of the meiotic cells, which appears to be of importance in connexion with the general mechanism of fibre formation (figures 5, 6, plates 5, 6).

(i) At the pachytene stage of meiosis a number of particles can be seen in the cytoplasm, if the analyzer of the interference microscope is set to show them up in preference to the nucleus (figure 5a, plate 5).

(ii) At the diakinesis stage, the particles are seen to have aggregated to produce short flexible fibres (figure 5b).

& Butler 1953). They are extremely sensitive to small changes in the electrolytic state of the medium. A change of this kind or an increase of concentration due to synthesis could account for the appearance of the more coherent structures seen during division. With chromosome threads which are formed by weak association between particles, the interchange between them which occurs at the end of pachytene (figure 4f, plate 4), can also be understood. Darlington has pointed out that this exchange takes place at the four-stranded stage, i.e. after synthesis of the individual thread is complete.

A mechanism for fibrogenesis, based on a process of linear aggregation, has been proposed by Astbury & Lomax (1934). Farrant, Rees & Mercer (1947) put forward a model of this kind for keratin, based on electron microscope studies of wool fibres. Randall (1953) has put forward a similar mechanism to account for collagen formation. The cytoplasmic structures which form the tail element in meiosis appear to be produced by such a process. Bélar (1929) examined this system as far as the end of the second anaphase. His material was treated with 5N-Ringer solution and fixed with osmic acid vapour. He showed that the fibres developed from cytoplasmic particles which were visible at late pachytene. These fibres appear to contract reversibly by a process of spiralization (figure 6, plate 6), again suggesting an analogy with chromosome behaviour. It appears that there may be physical similarities in the basic mechanisms of fibrogenesis and fibre behaviour in a number of cellular systems.

In conclusion it may be pointed out that the spontaneous formation of organized structures in solution will be favoured by the following conditions:

(1) The presence of moderately large and compact particles which will not be affected unduly by Brownian movement.

(2) Weak forces of interaction between the particles which will enable the equilibrium state to be reached by rearrangements under almost reversible conditions.

These properties appear to be well developed in nucleo-proteins.

SUMMARY

1. Unlike the fibres usually produced with synthetic materials, orientated fibres can be formed by biological systems without the application of external work. With calf thymus nucleo-protein, this occurs by an aggregation process when a critical concentration of the solution has been reached. These properties of fibre formation are not shown by nucleic acid alone.

2. In order to examine these phenomena in more detail a new technique has been worked out for the measurement of cell thickness, phase retardation and hence of concentrations of substances of high molecular weight within various regions of the living cell, which in the case of the nuclear threads are assumed to be nucleo-protein. Such measurements indicate that during meiosis, the concentration of such substances within the nucleus is always higher than the minimum value required to produce fibre formation in the model system of nucleo-protein, which has been studied.

3. With the interference microscope threads can in fact be seen in the resting nuclei of living cells in meiosis of *S. gregaria* throughout the cell cycle.

at which a marked degree of association occurs in solutions of calf thymus nucleo-protein. The presence of salt in the living nucleus will in fact lead to association at an even lower concentration (Ambrose & Butler 1953). Allowance must be made for the difference between mammalian and insect tissues, but measurements have shown that the concentrations in insect cells are in fact lower than those of mammalian cells. It is therefore likely that organized structures will be present within the nucleus at all stages of the cell cycle.

Evidence in favour of this view is given by the photographs shown in figure 4, plate 4. Interference microscopy provides a system of much greater sensitivity than any previously known for detecting small changes of refractive index in an inhomogeneous medium (Ambrose 1948). In figure 4, thread-like structures can be seen within the nuclei of all cells.

Various other tissues of *Schistocerca* have also been examined in collaboration with Dr A. R. Gopal-Ayengar. When favourable optical conditions have been obtained, thread-like structures have been seen in all nuclei. *S. gregaria* provides a particularly favourable case for observing threads in the cell nuclei, because the chromosomes are large.

It is of considerable interest to note that Japanese workers (Fujii 1926; Kuwada 1937; Kuwada, Sinke & Nakagawa 1939) found, as a result of their studies of living plant material, that the visibility of threads within the resting nucleus was simply dependent upon the salt concentration of the surrounding medium. They concluded that the density of the threads was dependent upon their degree of hydration. The difficulty experienced by recent workers with animal cells has probably been due to the small size of the chromosomes. Apart from problems of fixation, the electron microscope in particular is probably too insensitive to detect these small differences between the density of the threads and the nuclear sap, which can be seen, however, in the interference microscope.

As indicated in table 1 the concentration of the disperse phase (chromosome phase) increases in the nucleus as the cell comes towards division. This change is accompanied by increased clarity of the structures. It is also accompanied by spiralization which leads to longitudinal contraction. After the first meiotic division, the concentration in the interphase remains high (22 %). The second meiotic division is believed to follow without further synthesis of chromosome material. It is possible that the concentration within the nucleus may be of more relevance to the process of cell division than the total content (mass) of nuclear material.

Darlington (1937) has considered in some detail the role of structures similar to liquid crystals in relation to the behaviour of dividing cells. According to the balance theory of mitosis the chromosomes are looked upon as electrically charged bodies, whose arrangement at various stages of the cycle depends upon the magnitude and distribution of the charges, particularly those associated with the centrosomes and centromeres. The spindle is also considered to be a loose arrangement of particles whose cohesion increases as the cell divides. The experiments described in the present paper suggest that a similar physical picture may explain the behaviour of the chromosomes themselves during their development within the resting nucleus. Nucleo-proteins are known to be negatively charged (Ambrose

AN INTERFEROMETRIC METHOD OF QUANTITATIVE ANALYSIS OF ANTIGEN-ANTIBODY REACTIONS IN GELS

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INTRODUCTION

The most sensitive technique available for analysing complex mixtures of proteins consists of using these substances, by injection into a suitable animal, as antigens, and allowing them to diffuse through a gel containing the antibodies produced by the animal. Each antigen and its specific antibody combine in certain proportions, and if they are present in sufficient amounts a band of precipitate is formed.

Oudin (1946) allowed one reactant to diffuse into a gel in which the other was uniformly distributed. Ouchterlony (1949) and Elek (1948) allowed both components to diffuse towards one another through a gel. Complex mixtures of antigens and antibodies should be capable of analysis by means of these techniques, since each specific antigen-antibody system behaves independently as if the others were not present.
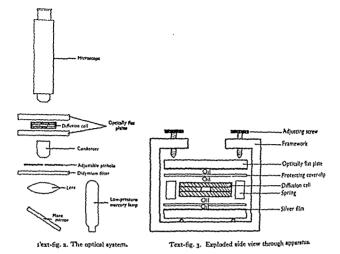
The principles of the interferometric technique have been described by Ambrose (1948), and a similar technique has been used by Robinson (1950) to examine the diffusion of solvents through polymers. It consists essentially of observing the diffusion of the reactants through a column of gel by means of an interference system of the Fabry-Pérot type derived from the multiple reflexions of a beam of light between two reflecting surfaces inclined to form a wedge (Text-fig. 1a). Interference fringes are observed, each fringe being a contour line of constant optical path. As diffusion of antigen or antibody proceeds a change of refractive index occurs and the initially straight fringes bend, each fringe representing exactly the refractive index gradient of the diffusing substance. If there is an accumulation of a complex between an antigen and its specific antibody in a particular region, an inflexion in the fringes occurs (Text-fig. 1b) in which the peak corresponds to the point of equivalence of the antigen and antibody. The quantity of antigen and antibody localized before commencement of precipitation may be measured directly from the area under the peak and the thickness of the gel. The technique is applicable to extremely complex mixtures of antigens and antibodies, and is also a very sensitive method of qualitative analysis.
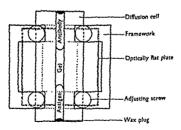
4. The formation of fibres by an aggregation process can also be seen to occur in the cytoplasm, during the formation of the secondary spermatocyte and immature sperm. These fibres also show spiralization phenomena which are analogous to chromosomes. Spiralization may in fact provide the simplest mechanism for the contraction, as the result of cohesive forces, of any fibre of moderate rigidity.

REFERENCES

Adair, G. S. & Robinson, M. L. 1930 *Biochem. J.* 24, 993.
Ambrose, E. J. 1948 *J. Sci. Inst.* 25, 134.
Ambrose, E. J. & Butler, J. A. V. 1953 *Disc. Faraday Soc.* 13, 261.
Ambrose, E. J., Cuckow, F. W. & Gopal-Ayengar, A. E. 1954 The fine structure of cells. *Proc. Int. Cell. Biol. Conf., Leiden*, p. 191.
Ambrose, E. J. & Gopal-Ayengar, A. R. 1952 *Nature, Lond.*, 169, 652.
Ambrose, E. J. & Gopal-Ayengar, A. R. 1953 Supplement to *Heredity*, 6, 277.
Astbury, W. T. & Lomax, R. 1943 *Nature, Lond.*, 133, 795.
Avery, O. T., MacLeod, C. M. & McCarty, Maclyn 1944 *J. Exp. Med.* 79, 137.
Barer, R. 1952 *Nature, Lond.*, 169, 366.
Barer R. & Tkaczyk, S. 1954 *Nature, Lond.*, 173, 821.
Bélar, K. 1929 *Arch. EntwMech. Org.* 118, 359.
Bernal, J. D. & Fankuchen, I. 1937 *Nature, Lond.*, 139, 923.
Darlington, C. F   ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ 2nd ed. London
Davies, H. S. &   ⋯   *Biol. 'Physical Aspects of*   541.
Dyson, J. 1949 *Nature, Lond.*, 164, 229.
Easty, G. C. & Ambrose, E. J. 1957, *J. Exp. Biol.* 34, 60.
Easty, D. M., Ledoux, L. & Ambrose, E. J. 1956 *Biochem. biophys. Acta*, p. 528.
Elliott, A. & Ambrose, E. J. 1950 *Disc. Faraday Soc.* 9, 246.
Farrant, J. L., Rees, A. L. & Mercer, E. H. 1947 *Nature, Lond.*, 159, 535.
Fujii, K. 1926 *Dept. Jap. Ass. Adv. Sci.* 2 (Kyoto Meeting); *Toyogakugeiz Assi.* 43.
Jackson, S. Fitton 1956 *Proc. Roy. Soc.* B, 144, 556.
Kuwada, Y. 1937 *Cytologia, Tokyo*, Fujii Jub. No., p. 389.
Kuwada, Y., Sinke, N. & Nakagawa, Z. 1939 *Cytologia, Tokyo*. 9, 393.
Mazia, D. 1954 *Proc. Nat. Acad. Sci., Wash.*, 40, 521.
Mellors, R. C., Kupfer, A. & Hollander, A. 1953 *Cancer*, 6, 372.
Randall, J. T. 1953 *The nature and structure of collagen*, p. 232 (Butterworths).
Robinson, Conmar 1950 *Proc. Roy. Soc.* A, 204, 359.
Shooter, K. V., Davison, P. F. & Butler, J. A. V. 1954 *Biochem. biophys. Acta*, 13, 192.
Smith, F. H. British Patent Spec. 639014.
Tolansky, S., 1948 *Multiple beam interferometry of surfaces*. Oxford: Clarendon Press.
Yasuzumi, S., Odate, F. & Ota, Y. 1951 *Cytologia, Tokyo*, 16, 233.

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Text-fig. 2. The optical system.

Text-fig. 3. Exploded side view through apparatus.

Text-fig. 4. Top view of apparatus with upper optical plate removed.

Text-fig. 1. (a) Illustrating the principle of the method; and (b) indicating the interference fringes in the same orientation.

METHODS

The interference system consists of two ¼ in. thick glass plates (2 by 1½ in.). Plates of this thickness are used in order to prevent distortion of the fringes when the plates are subjected to the slight pressure which it is necessary to apply to adjust the angle between them. Each plate is optically flat on one surface which is silvered *in vacuo* to give about 10% transmission of white light. The density of the silvering is important since if the film is too dense, insufficient light is transmitted, and if the film is insufficiently dense the lack of contrast in the interference fringes makes observation difficult. The silver film is very easily damaged and deteriorates if exposed to the atmosphere for too long. To overcome these difficulties the silvered surfaces can be protected with large cover-slips cut to size and held on the silvered surface by a thin film of immersion oil. The protection can be made even more secure by sealing the edges of the cover-slip and plates with a cold-setting resin such as 'Araldite'. Protected this way and used carefully, one pair of silvered optical flats will last almost indefinitely. Thin cover-slips were used to protect the silver film rather than thicker glass plates, since the contrast of the fringes decreases as the distance between the silvered surfaces increases, although the sensitivity is increased at the same time.

The apparatus is shown diagrammatically in Text-figs. 2–4.

RESULTS

Two main methods have been used by previous workers to characterize antigens present in a mixture. The first, developed by Oudin (1947, 1948 a, b, 1949), consists of allowing the antigenic mixture to diffuse into antiserum which has been set in agar, when a number of precipitation fronts are developed. The distance travelled by these fronts depends on the concentration of the antigenic components, on their diffusion coefficients and on the square root of the time. The diffusion coefficients can be determined from the diffusion test and thus the quantities of antigens may be determined. Becker (1953) and other workers, however, claim that the presence of non-reacting substances can lead to serious errors in the estimation, mainly due to the effect they have on the migration rates of the bands.

The second method, developed by Oakley & Fulthorpe (1953), uses a double diffusion technique. Antiserum is set in agar in the bottom of a tube; on this is placed a central column of agar on which the antigenic mixture is poured. Disks of precipitate are formed in the agar column. If an antigen is diluted its disk appears higher in the column and is fainter. The amounts of antigen present in the mixture may be roughly estimated by finding the smallest amount of it which, when allowed to diffuse through constant antiserum, gives a line in the upper meniscus of the central agar column. Presumably the system is first calibrated by using a solution containing a known concentration of the antigen. Furthermore, the antigens may be characterized by the values of antisera against them, determined by finding the amounts of antisera it is necessary to add to the antigenic mixture to move each line in turn into the upper meniscus, or to cause it to fade out. With mixtures giving a few lines, Oakley has achieved considerable accuracy with this technique, but the more complex the mixture the more difficult it is to apply.

QUALITATIVE APPLICATIONS

Qualitatively, the interferometric method is superior to the techniques at present used to detect the number of bands or fronts formed, particularly when the accumulation of antigen-antibody complex has been too small to result in the formation of a precipitate. This occurs most frequently with 'flocculating' systems but also in 'precipitating' systems. Such regions can often be picked out as Schlieren lines without the aid of the interferometer but if there are several of them close together it is generally impossible to count the number. Pl. 1, fig. 3, shows such a system, which when viewed suitably with the naked eye or ordinary microscope showed a broad region obviously containing a large number of lines which contained no detectable precipitate. It was impossible, however, to form an accurate estimate of the number present Viewing through the interferometer immediately revealed a large number of small but definite peaks in the fringes, which cannot be caused by an irregular inhomogeneity in the gel as the peaks in the fringes are superimposable, showing that the inhomogeneity runs right across the width of the channel at right angles to the direction of diffusion. This can be seen more clearly in the tracing of

The two plates with their silvered surfaces facing one another are placed horizontally on a microscope stage separated by two narrow strips of sponge rubber or thin steel springs leaving a gap of 2–3 mm. The plates are mounted in a rigid brass framework which has four vertical screws which may be screwed down on to the four corners of the uppermost plate, thus permitting the delicate adjustment of the angle between the plates on which the spacing of the fringes is dependent. The object to be viewed is placed between the plates with a film of immersion oil between the object and the plates. The plates are adjusted until they are almost parallel. They are illuminated from below by a beam of parallel monochromatic light from a low-pressure mercury vapour lamp. For photography it was found advantageous to insert a Kodak Didymium filter. The angle of incidence of the monochromatic light, on which the sharpness of the fringes is very dependent, must be adjustable by means of a horizontally movable pin-hole. Very sharp Fabry-Pérot-type fringes are obtained on a practically dark field, each fringe being a contour line of constant optical path between the silvered surfaces of the plates. The wedge angle between the plates is usually adjusted to be at right angles to the direction of diffusion, and as diffusion proceeds there is a displacement of the fringes to maintain the optical path constant.

The diffusions were generally carried out in cells constructed from three microscope slides. One slide was cut along its length into two halves which were placed between two whole slides to form a flat tube about 4 mm. wide, 1 mm. deep and 7 mm. long, open at both ends. For ordinary purposes the best seal was obtained by dipping the edges of the cell, held firmly in a bulldog-clip, into a dish of molten paraffin wax containing an equal quantity of vaseline. It was found that a strong watertight seal was obtained without any wax penetrating into the diffusion channel. If microscope slides having obvious optical flaws were rejected it was found that the slides were sufficiently flat over the small areas utilized to give no distortion of the interference fringes. The molten gel was then injected into the centre of the channel using a hypodermic syringe, care being taken to avoid the formation of bubbles which is not difficult if the channel is free of grease. When the gel had set the antigen solution was injected into the space at one end of the column of gel and the antibody solution at the opposite end. The ends of the channel were then sealed with a little of the molten paraffin wax/vaseline mixture.

The use of gelatin presents no difficulties as it adheres strongly to glass, but agar contracts on setting and often a leakage occurred along one or more of the agar/glass interfaces. This can be overcome in the ordinary gel-diffusion technique by drying a film of agar gel on to the glass before adding the agar diffusion gel. When this was tried it was found that the resulting column of gel, although homogeneous to the naked eye, was far from being so under the interferometer. One way of overcoming this leakage difficulty using agar gel was to allow the agar to set and then to inject gelatin solution into the spaces at either end of the agar column. The excess gelatin solution was carefully drained off using filter paper, and the remainder which wets all the surfaces was allowed to set, thus sealing all the potential leaking interfaces. The thin film of gelatin was found not to retard diffusion to any detectable extent.

fig. 1) since no precipitate is formed until the ratio of antigen to antibody rises to a certain minimal level. Such systems are horse antisera against certain proteins such as diphtheria toxin, serum albumins, ovalbumin and conalbumin. With the 'rabbit' (precipitating) systems the peaks are not so large as precipitate is formed however high the ratio of antigen to antibody may be; and precipitated complex appears as a more or less opaque region in the interferometer. With both systems, once the precipitate has been formed it does not contribute to the refractive index in that region to the extent which it would in the absence of precipitate formation. There are two procedures for overcoming this difficulty.

(1) *Dilution of reactants*

The antigen and antibody solutions are allowed to diffuse through the gel for a certain period and interact. The areas under any observable peaks as in Pl. 1, fig. 1, are measured and the quantities present calculated. By diluting the antigen and antibody solutions to the same extent any dense bands will diminish and eventually give measurable peak areas. The positions of the bands should be unchanged as both the antigen and antibody solutions are diluted to the same extent. From the measured areas under the peaks at different dilutions the quantity of each antigen-antibody complex can be calculated.

(2) *Dissolving the precipitates*
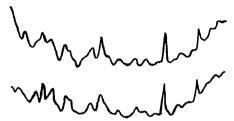
Turner & Boyer (1952) have shown that the antigen-antibody precipitates may be completely dissolved at pH 11 or above, forming large aggregates with particle sizes about × 250 the diphtheria antitoxin molecule in the case of the diphtheria toxin-antitoxin complex. Some loss of serological activity occurs but this should have little effect, if any, on the refractive index of the proteins concerned. Kleinschmidt & Boyer (1952) found that a solution of sodium dodecyl sulphate was the most active reagent for dissolving the complexes and this was found to be true also for the complexes formed in the gels. Unfortunately, sodium dodecyl sulphate dissolves gelatin most effectively, so this reagent can only be used with agar gel. It was found, however, that provided the pH was kept below 13 units and the gelatin was not too weak the bands of precipitate could be dissolved without dissolving the gelatin. Both sodium dodecyl sulphate and alkaline solutions may be used for dissolving bands of precipitate formed in agar. Two techniques were developed for dissolving the bands.

(a) *By diffusion of reagent from the antigen or antibody wells*

This could be applied to both gelatin and agar columns. The wells at either end of the diffusion column were drained and filled with the reagent. The reagent slowly diffused and dissolved the bands of precipitate. The stages in the dissolution of an opaque band of precipitate with the formation of a peak is illustrated in Pl. 2, figs. 3–6. The peaks gradually become broader and flatter as the particles of complex diffuse away. This process may take from a few minutes to several hours depending

the contours of the fringes shown in Text-fig. 5. The multiplicity of the main opaque band on the right of Pl. 1, fig. 3, can be seen in the interferometer but not with the ordinary microscope. Occasionally, very faint diffuse bands were encountered in 'precipitating' systems which were just detectable to the naked eye but did not appear as peaks in the fringes, presumably because they were not contributing sufficiently to the refractive index. Such bands are easily detectable, however, by adjusting the angle of the incident beam so that the fringes almost vanish.

Text-fig. 5. Tracing of the contours of the fringes shown in Pl. 1, fig. 3.

The increased sensitivity of the interferometer over other methods of examination is demonstrated by the observation that the main band of the diphtheria toxin-antitoxin system could be resolved into at least six razor-sharp interference peaks within the narrow band of precipitate, which appears to be single and homogeneous under the ordinary microscope. This is in agreement with the observation of Pope (1954) that crystalline diphtheria toxin contained at least six antigens, although it gave a single line in the diffusion test. Pl. 1, fig. 4, considerably enlarged and taken with the fringes almost completely spread out, shows the complexity of this main band.

QUANTITATIVE APPLICATIONS

The quantitative applications of the method are all based on the measurement of the areas under the peaks formed in the interference fringes by the accumulation of antigen-antibody complex. Fortunately, the refractive indices and specific refractive increments are almost constant for all proteins (Adair & Robinson, 1930), hence the relative and absolute amounts of protein present in each peak in the diffusion cell may be measured. This will be roughly true even for non-protein antigens since the polyvalent antigens generally combine with considerable excess of antibody to form the complex at equivalence point. The measurement of the areas under the peaks is greatly simplified by the use of the camera lucida. Pronounced peaks are easily observed with 'horse' (flocculating) systems (see Pl. 1,

Suppose that the width of the channel is w cm. and that the extreme edges of the zone of precipitation are at points l_1 and l_2 along the length of the channel.

$$\text{Total mass in the reaction zone} = \int_{l_1}^{l_2} \left(\frac{\mu_c - \mu_m}{\alpha} \right) tw\, dl$$

$$= \int_{l_1}^{l_2} \frac{n_c \lambda}{\alpha 2t} tw\, dl$$

$$= \frac{\lambda w}{2\alpha} \int_{l_1}^{l_2} n_c\, dl.$$

$\int_{l_1}^{l_2} n_c\, dl$ is simply the area measured under the peak expressed in fringe displacement along the length of the channel.

A measurement of this quantity indicates the total mass of antigen-antibody complex formed within the reaction zone.

There are errors inherent in both these procedures, but they should be less than those mentioned by Becker (1953) in estimations by Nordstrom in his laboratory of bovine serum albumin with rabbit anti-bovine serum albumin using the Oudin technique, when errors of 50–60% were encountered.

GENERAL OBSERVATIONS

We have obtained indications that when a band of precipitate becomes too dense it can act as a barrier, since the concentration of substances in solution on one side of the band has been found to be higher than on the other side. It should be emphasized that this has only been observed in a few extreme cases. It is probable that beyond a certain critical density the band of precipitate may become virtually impermeable to components of very high molecular weight. Similarly, with very dense bands the structure of the supporting gel appears to be disrupted where the band is formed since the column of gel will break exactly at that point if gently agitated in water. With care the thin sheet of precipitate may be separated quite sharply from the gel on either side by this treatment.

The interferometric observations are in agreement with the principle of Goldberg (1952) that the aggregates of antigen and antibody will continue to grow up to a critical point at which a few large aggregates will be formed by the union of numerous aggregates of moderate size, which then precipitate. Most complexes may be detected as peaks in the interference fringes before any precipitate is formed. These peaks do not diffuse away if the antigen and antibody solutions are withdrawn, and must therefore be in the form of aggregates too large to diffuse through the interstices of the gel but not sufficiently large to form a visible precipitate.

The examination of the diffusion gradients of the antigen or antibody solutions alone in the absence of the other reactant has not revealed any indication of the absorption of any component by the gel, whether agar or gelatin. Diffusion is much slower through gelatin and the resulting bands and peaks appreciably sharper. In using the double-diffusion technique it has also been observed that if the antigen

on the strength of the reagent at the site, and the nature and quantity of the precipitate present. Unfortunately the presence of a diffusion gradient of the reagent can sometimes obscure the peaks obtained and to try to avoid this the second technique was developed.

(b) By diffusion of the reagent from the side of the diffusion column

This technique has only been tried with agar gel owing to the difficulties encountered in exposing the side of a gelatin column without distortion of the gel and hence of the fringes. The glass cell is split with the blade of a scalpel and the column of gel is floated off the microscope slide into a solution of the reagent, and then quickly placed between the interference plates, the air gap between the column of gel and the plates being filled with a thin film of reagent solution. Effective diffusion appears to take place mainly from the exposed surfaces where the gel is in contact with a large volume of reagent solution. The bands dissolve as illustrated in Pl. 2, figs. 1, 2. The advantage of this technique over the first one is that all bands are dissolved simultaneously, the reagent diffusion gradient being across the width of the cell instead of along its length and thus each band should be dissolved in the same gradient and any distortions are the same for each peak obtained. The disadvantage is that the peaks appear to be broader and flatter than those obtained by the first technique.
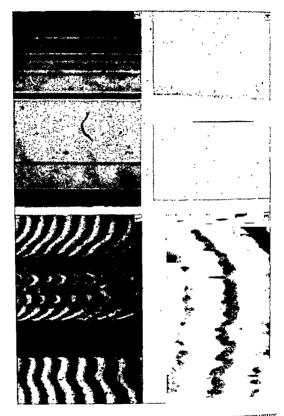
Both these methods possess an advantage over the dilution of reactants technique in that it is possible to obtain a measure of the quantities present in each band using a single diffusion cell, which need only contain 0.1–0.2 ml. of antigen and antibody solution. Generally, weak bands of precipitate are obliterated before the transient peak areas can be measured, and in practice it is preferable to combine both the dilution and dissolution techniques, i.e. measure the areas under the peaks where the precipitate is not dense enough to interfere, then dissolve the bands and estimate the quantity present in each of the peaks resulting from the dense bands. In order to determine the total mass of complex in a given band the displacement of the fringes in a direction perpendicular to the length of the channel should be measured, the fringes in the clear medium being set to run parallel to the length of the channel.

Let us consider a particular point in the reaction zone (distance l cm. along the channel) where the shift relative to the clear medium is n_e fringes (expressed in whole fringes plus any fractional shift). The retardation of the light beam is then equal to $n_e\lambda$ cm., where λ is the wave-length of mercury green radiation. Suppose that the refractive index of the medium at this point is equal to μ_e while that of the clear medium is μ_m. If t cm. is the cell depth

$$2t\,(\mu_e - \mu_m) = n_e\lambda.$$

The specific protein increment (α) is the increase of refractive index for the addition of 1 g. of protein to 100 ml. of medium (0.00185 for most proteins). If x g./100 ml. is the concentration of complex at the given point in the reaction zone

$$x = \frac{\mu_e - \mu_m}{\alpha}.$$

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and antibody wells are at least three to four times larger in volume than the column of gel between them, the bands of precipitate and peaks remain stationary once they are formed indicating that a steady state has been formed.

It is hoped that the technique may prove of general application, particularly to a study of the complex mixtures of proteins associated with problems of tissue specificity.

SUMMARY

1. A sensitive interferometric method of observing antigen-antibody complexes formed by diffusion of the reactants through gels in suitable diffusion cells is described.

2. Bands of antigen-antibody complex, if not too dense, are observed as peaks in the interference fringes, the areas under the peaks being proportional to the quantities present in each band.

3. The method may be made quantitative by measurement of the areas under the peaks of bands in which precipitation has not progressed too far, and by dissolving the bands of dense precipitate by diffusion of a suitable reagent to give peaks in the fringes.

4. An equation expressing the quantity of precipitate present in a band has been derived.

REFERENCES

ADAIR, G. S. & ROBINSON, M. E. (1930). The specific refraction increments of serum-albumin and serum globulin. *Biochem. J.* 24, 903
AMBROSE, E. J. (1948). An interference microscopy for the study of inhomogeneous media. *J. Sci. Instrum.* 25; 134.
BECKER, E. L. (1953) Antigen-antibody reactions in gels *Fed Proc* 12, 717
ELEK, S. D. (1948). The recognition of toxicogenic bacterial strains *in vitro*. *Brit. Med. J.* 1, 493.
GOLDBERG, R. J. (1952) A theory of antigen-antibody reactions. I Theory for reactions of multivalent antigen with bivalent and univalent antibody *J Amer Chem Soc.* 74, 5715.
KLEINSCHMIDT, W. J. & BOYER, P. D. (1952) Dissociation studies with egg albumin—anti-egg albumin precipitates. *J Immunol* 69, 257.
OAKLEY, C. L. & FULTHORPE, A. J. (1953) Antigenic analysis by diffusion. *J Path. Bact.* 65, 49.
OUCHTERLONY, O. (1949). Antigen antibody reactions in gels *Acta Path. Microbiol* 26, 507
OUDIN, J. (1946) Méthode d'analyse immunochimique par précipitation specifique en milieu gélité *C R Soc Biol.*, *Paris*, 222, 115.
OUDIN, J. (1947) L'analyse immunochimique du sérum de cheval par précipitation specifique en milieu gélifié Premiers résultants *Bull Soc. Chim. Biol*, *Paris*, 29, 140.

Oudin, J. (1948a). L'analyse immunochimique qualitative; méthode par diffusion des antigènes au sein de l'immunsérum précipitant gélosé. *Ann. Inst. Pasteur*, 75, 30.

Oudin, J. (1948b). L'analyse immunochimique qualitative; méthode par diffusion des antigènes au sein de l'immunsérum précipitant gélosé. *Ann. Inst. Pasteur*, 75, 109.

Oudin, J. (1949). La diffusion d'un antigène dans une colonne de gel contenant les anticorps précipitants homologues. Etude quantitative des trois principales variables. *C.R Soc. Biol., Paris*, 228, 1890.

Pope, C. G. (1954). The diphtheria antigen-antibody flocculation system. *Disc. Faraday Soc.* 18, 323.

Robinson, C. (1950). Interferometric studies in diffusion. I. Determination of concentration distributions. *Proc. Roy. Soc.* A, 204, 339.

Turner, E. W. & Boyer, P. D. (1952). Dissociation studies with diphtheria toxoid-antitoxin precipitates. *J. Immunol.* 69, 265.
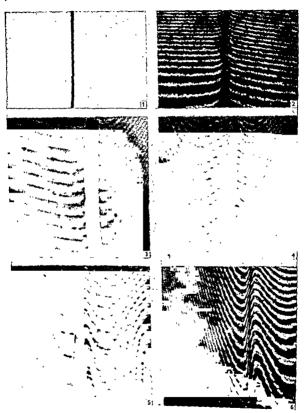
EXPLANATION OF PLATES

Plate 1

Fig. 1. Diphtheria toxin-antitoxin system in the interferometer, showing interference fringes and one opaque band of precipitate; also a number of peaks where precipitation either has not occurred or has just commenced.

Fig. 2. Another diphtheria toxin-antitoxin system in the interferometer with the interference fringes almost completely obscured so that only visible bands of precipitates are detectable.

Fig. 3. A rat serum-antirat serum system showing numerous small peaks indicating the presence of many complexes, most of which had not formed any visible precipitate. The group of fine dark bands on the right-hand side show the multiplicity of a band of precipitate which appeared single under the ordinary microscope.

Fig. 4. A greatly enlarged photograph of the main band from the diphtheria toxin-antitoxin system taken with the interferometer using a large pinhole and the fringes almost completely obscured. Six or more components may be seen. This effect could only be detected at an early stage in the development of the band and with a considerable distance between the toxin and antitoxin wells.

Plate 2

Fig. 1. Opaque band of precipitates before dissolution.

Fig. 2. Same band showing fringes and formation of a peak on dissolution of the band of precipitate by diffusing reagent into the side of the column of gel. In the photograph diffusion is taking place from the upper edge.

Fig. 3. A band of precipitate showing fringes before dissolution of the precipitate.

Fig. 4. The same band with reagent diffusing from the left. The opaque band has almost completely dissolved producing a marked inflexion of the fringes.

Fig. 5. The same band 5 min. later. The band has completely dissolved and a single fringe may be followed through from one side of the peak to the other.

Fig. 6. The same band 10 min. after fig. 5. The peak is shorter and broader as diffusion of the dissolved and dissociated antigen-antibody complex proceeds.

EASTY and AMBROSE—AN INTERFEROMETRIC METHOD OF QUANTITATIVE ANALYSIS OF ANTIGEN-ANTIBODY REACTIONS IN GELS

Imagawa, Syverton and Bittner (1954) showed that antisera to mouse mammary cancer cells were cytotoxic to the tumour cells. The elimination by repeated adsorption of the antigenic components which were possessed in common by normal cells and cancer cells from the same host species was without apparent effect upon the specificity of the cytotoxic antiserum.

In the work reported here cell suspensions were also used to investigate the cytotoxicity of the antisera. A gel-diffusion technique based on those developed by Oudin (1946) and Ouchterlony (1948) was employed to detect differences in the composition of the soluble antigens of the tumours cells and the normal cells of the host. It is certain that not all the antigens present in a cell can be detected using the gel-diffusion technique because of the presence of some of the antigens in very small quantities and the presence of others in a non-diffusable particulate or fibrous form. It was thought that the study of the effect of the antisera on the living cells *in vitro* might prove useful for detecting antigens not revealed by the gel-diffusion technique, in particular those antigens present in the cell membrane.

<h2 style="text-align:center">MATERIALS AND METHODS</h2>

(a) *Transplantation of tumours.*—The Ehrlich and Landschütz ascites tumours were propagated in the C — and C + strain mice respectively. The tumours were propagated by injecting 0·3–0·4 ml. of the ascitic fluid intraperitoneally into mice of the appropriate strain. The Ehrlich ascitic fluid was frequently haemorrhagic, whereas the Landschütz fluid was generally cream in colour and contained relatively few red blood cells.

(b) *Preparation of antisera.*—For immunization the ascitic fluids were withdrawn using a syringe, washed with calcium-free citrated Locke's solution and most of the cells collected by gentle centrifugation. This was repeated five or six times. The use of the calcium-free citrated Locke's solution was found to reduce considerably the clumping of the cells which took place on centrifugation and which interfered with the efficiency of the washing. The red blood cells were removed as much as possible with the supernatant fluid, and suspensions of Landschutz ascites cells were obtained which after five washings contained less than one red blood cell to fifty tumour cells. Provided the washing procedure was carried out quickly and centrifugation was gentle, most of the tumour cells appeared to remain intact. Rabbits were immunized by intravenous injections both of intact and homogenized ascites tumour cells twice a week for four weeks. The sera obtained after bleeding the rabbits appeared to possess approximately the same immunological properties and titre regardless of whether the cells injected were intact or homogenized. One rabbit was immunized by subcutaneously injecting cells which had been emulsified with Arlacel A and Bayol F.

(c) *Preparation of cell suspensions.*—The preparation of tumour cell suspensions naturally presented no difficulty. The cells were washed once with calcium-free citrated Locke's solution and then two or three times with calcium-free Locke's solution containing no citrate. The preparation of suspensions of normal cells of the host mice in an intact condition presented much greater difficulty. The mice were perfused through the aorta with citrated calcium-free Locke's solution. The liver, kidney and spleen were removed, finely chopped and gently shaken in tubes containing calcium-free Locke's solution. The suspensions were allowed to settle for a few minutes and the supernatant fluid containing varying

THE ANTIGENIC COMPOSITION OF MOUSE ASCITES TUMOUR CELLS USING *IN VITRO* AND GEL-DIFFUSION TECHNIQUES

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ONE of the main problems encountered in an investigation of the possible antigenic differences distinguishing normal and tumour cells is the difficulty of obtaining " pure " suspensions of tumour cells essentially free of contaminating normal cells for immunization. There appear to be three ways of obtaining cells in this condition : tissue culture, ascites tumours and cell suspensions made from solid tumours. It is difficult to obtain suspensions of tumour cells from solid tumours which are free of normal cells and in reasonably high yield, whereas ascites tumour cells can be obtained in large quantities virtually free of all normal cells except blood cells, most of which can be removed by careful centrifugation. Klein (1956) has stated, " In typical ascites tumours the percentage of neoplastic cells is very high and represents a nearly pure culture ".

For these reasons it was decided to investigate using immunological techniques any antigenic differences that might exist between mouse ascites tumours and the normal cells of the host. Genetic differences would certainly exist between the tumours and the normal cells of the host and it would be of interest to see if the techniques employed would demonstrate such differences unambiguously.

Various workers have prepared antisera which they claimed showed varying degrees of specificity for the tissue injected, whereas others claim to have found little evidence for specificity beyond species specificity. Verne and Oberling (1932) claimed that rabbit antirat kidney serum damaged epithelium and fibroblasts of kidney in tissue culture but had no effect on cells from other organs. Harris (1943) found compete species specificity with antisera prepared by injecting chick and mouse tissues into rats, but only partial organ specificity. Lambert (1914) injected three guinea-pigs each with a different rat tissue and found that the antiserum from each rat overlapped. Brosman and Korngold (1955) found that . . . . They also showed that by *in vitro* purification some of the tumour localizing antibodies could be partially separated from the liver and kidney localizing antibodies. Maculla (1948) concluded from work on the comparative antigenic composition of homologous and heterologous mouse tumour transplants that all the tumours appeared to possess intracellular antigenic compositions in common with adult spleen and lung, but some tumours appeared to possess a component not present in normal tissue. Schrek and Preston (1956) have shown that sera from rats with regressed lymphosarcoma have an antibody against the cells of this tissue. These workers used cell suspensions to demonstrate the cytotoxicity of their sera

the normal tissue cells. At 37° the tumour cells in the presence of a mixture of immune serum and normal rabbit serum underwent lysis within 5 minutes with no cells surviving except on rare occasions when a few cells survived for half-an-hour. Normal liver cells would survive under these conditions for about an hour before lysis became markedly apparent. In normal rabbit serum the viable tumour cells, if unflattened, presented a characteristic appearance in the interference microscope. With the interference contrast adjusted to give a bright field the periphery of the cell was marked by a narrow, well-defined dark ring with little structure visible inside the cell (Fig. 1). Generally the outer margin of the cells showed the undulating activity of the membrane which can be observed very clearly using the interference microscope. When the immune serum was introduced the tumour cells rounded up immediately and the dark, well-defined periphery began to extend inwards from the edge of the cell (Fig. 2). The freely moving cells quickly aggregated into clumps and within a few minutes blisters of clear cytoplasm containing few visible granules were formed on the surfaces of the cells (Fig. 2). As the degenerative changes proceeded and the lysis developed the nucleus became more prominent, and structures within it and the cytoplasm became apparent. After 5 to 10 minutes the cytoplasm was observed to have almost as low a refractive index as the medium surrounding the cells (Fig. 3). This occurred even with those cells which had not formed cytoplasmic blisters and thus not suffered a severe change in cell volume. This pronounced drop in the refractive index indicated that most of the cytoplasmic constituents had been lost. The membrane appeared to be intact at this stage, not breaking down and releasing the granular constituents of the cell until about 20 minutes after the addition of the immune serum The cell appeared to have reached an irreversible condition long before the cell membrane broke down. If cells were incubated with immune serum for 5 minutes and then washed to remove immune serum, the degenerative changes continued at a reduced rate, recovery of individual cells being rarely observed. The nucleus also lost material at a later stage when the chromatin granules became more clearly visible.

The effect of the immune serum on Ehrlich ascites cells was examined in greater detail using the interference microscope and time-lapse colour cinematography. The immune serum was drawn under the coverslip using filter paper whilst the cells were being filmed. The cells reacted immediately to the antiserum, expanding very quickly. This was followed after a short interval by a contraction in which the cell returned roughly to its original size. After another short interval the characteristic cytoplasmic blisters were formed, ballooning out from the surface of the cell. The whole process generally took less than 2 minutes with very active immune serum.

Similar effects were seen when suspensions of normal cells were treated with the immune serum, except that under comparable conditions the normal tissue cells took longer to lyse and did not usually form blisters of cytoplasm which was such a distinctive feature of the tumour cell death. The gradual loss of material from the cytoplasm together with the appearance of cytoplasmic granules and nuclear structure was observed, as with tumour cells. Aggregation of suspensions of normal cells did not occur to the same extent as with tumour cells with the exception of erythrocytes. Dilution of the immune serum by a factor of 200 still produced detectable cytolysis of the tumour cells but hardly at all with normal cells except erythrocytes.

numbers of individual and small clumps of cells were washed with calcium-free Locke's solution and concentrated by gentle centrifugation.

At room temperature and at 37° the tumour cell suspensions appeared to remain intact for far greater lengths of time than any of the normal cell preparations, whether suspended in Locke's solution or with normal serum added. For the in vitro tests the cells were ⋅ ⋅ ⋅ ⋅ ⋅ ⋅ ⋅ ⋅ ⋅ ⋅ ⋅ scope slide and the coverslip se ⋅ ⋅ ⋅ ⋅ ⋅ mixture and the cells examined ⋅ ⋅ ⋅ ⋅ ⋅ in a perspex box maintained at 37°. At this temperature it was found that most preparations of normal cells in Locke's solution or in normal serum began to show morphological changes preceding cell death after about half-an-hour and most of the cells did not survive more than 3 hours. Occasionally, preparations of normal cells were obtained in which groups of cells appeared to be intact after 6 hours. Tumour cell preparations, on the other hand, would often last for 24 hours under these conditions especially in the presence of added normal serum.

(d) *Absorption of immune sera.*—The immune sera were absorbed with blood and finely minced tissues of the host by incubating the tissues and the immune sera together at 37° for half-an-hour and then leaving at 0–4° for several days. The serum was then recovered by centrifugation and stored frozen. As far as possible, care was taken to avoid the addition of too great an excess of blood or tissues in order to reduce the possibility of non-specific absorption of antibodies.

Analytical methods

(a) In vitro *tests* —The interference microscope was used to examine the action of various antisera on normal and tumour cells *in vitro*. The interference microscope is particularly suitable for the detection of lysis of cells since quite small variations *in the mass of the cell constituents can be detected and irregularities in the* appearance of the cell membrane, which we found was generally the first symptom of cellular degeneration, are very clearly seen with the interference microscope. To a lesser extent the non-staining of viable cells by the dye Lissamine Green (private communication, Dr. R. J. Goldacre) was used to examine the cytotoxicity of the antisera.

(b) *Gel-diffusion tests.*—The presence of antibodies in the immune serum *to* soluble antigens obtained fom the cells was demonstrated using the gel-diffusion technique. In this technique the antigen and antibody solutions are allowed to diffuse towards one another through a column of gel. Bands of precipitate are formed, one band occurring at the equivalence point of each specific antigen antibody system, no individual antigen giving more than one band of precipitate

EXPERIMENTAL RESULTS

(1) In vitro *tests with the original immune sera*

Preliminary *in vitro* tests with all the original immune sera showed that all the sera were cytotoxic to the tumour cells and the cells of the normal liver, kidney, spleen and blood of the host, the strength of the reaction based on lysis times of the cells being in the order : tumour, erythrocytes, kidney, spleen, liver. The tumour cells lysed more rapidly and after a shorter interval of time than any of

TABLE I

In this table the survival times of the various tissue cells in the presence of
normal serum and anti-Ehrlich ascites tumour serum are briefly
summarized.

| | Time of lysis with added serum | | |
| | In normal serum | In normal serum + immune serum (mins) | In normal serum + absorbed immune serum |
Type of cell	In normal serum (hrs.)		
Ehrlich ascites tumour cells .	8–24	3–5	15–20 minutes
Landschütz ascites tumour cells	8–24	10–15	20–30 ,,
Normal kidney cells . .	1–3	10–30	1–3 hours
Normal spleen cells . .	1–3	20–40	1–3 ,,
Normal liver cells . .	3–12	40–60	3–12 ,,
Erythrocytes . .	8–24	5–10	8–24 ,,

4   *Gel-diffusion tests with absorbed immune sera*

These tests were carried out mainly with the Ehrlich ascites antiserum absorbed
. . . . !   With these absorbed sera the Ehrlich
(. . .   . .   the gel-diffusion test, and the Land-
:   . . . .   nt of 2 bands, but always one strong
band was missing in the Landschütz system that was present in the Ehrlich system,
all other bands being present in both in about the same quantities (Fig. 12).
This demonstrated the presence of at least one soluble antigen present in the
Ehrlich ascites tumour cells which was absent in the Landschütz ascites tumour
cells. The normal kidney, spleen and liver cells and blood gave no bands with the
absorbed Ehrlich ascites antiserum (Fig. 13–16) although faint non-specific

EXPLANATION OF PLATES

FIG.   *   .   .. . ' . . . .
FIG.   .   .   .   .   .   3 minutes.
FIG.   .   .   .   ·6 minutes.
FIG.   .   and normal
    guinea-pig serum, after 15 minutes.
FIG. 5.—Ehrlich ascites tumour cells in absorbed anti-Ehrlich immune serum and normal
    guinea-pig serum after 30 minutes.
FIG. 6.—Landschütz ascites tumour cells in absorbed anti-Ehrlich immune serum and normal
    guinea-pig serum after 25 minutes.
FIG. 7.—Landschütz ascites tumour cells in absorbed anti-Ehrlich immune serum and normal
    guinea-pig serum after 45 minutes.
FIG. 8.—Erythrocytes from C— mouse in absorbed anti-Ehrlich immune serum and normal
    guinea-pig serum after 4 hours at room temperature.
FIG. 9.—Kidney cells from C— mouse in absorbed anti-Ehrlich immune serum and normal
    guinea-pig serum after 1½ hours.
FIG. 10.—Spleen cells from C— mouse in absorbed anti-Ehrlich immune serum and normal
    guinea-pig serum after 1½ hours.
FIG. 11.—Liver cells from C— mouse in absorbed anti-Ehrlich immune serum and normal
    guinea-pig serum after 2 hours.
FIG. 12.—Gel-diffusion test. Landschütz tumour cells on left, Ehrlich on right, absorbed
    anti-Ehrlich immune serum at the bottom.
FIG. 13.—Gel-diffusion test Blood from C— mouse on left, Ehrlich tumour cells on right,
    absorbed anti-Ehrlich immune serum at the bottom.
FIG. 14.—Gel-diffusion test Minced kidney from C— mouse on left, Ehrlich tumour cells on
    right, absorbed anti-Ehrlich immune serum at the bottom.
FIG. 15.—Gel diffusion test. Minced spleen from C— mouse on left, Ehrlich tumour cells on
    right, absorbed anti-Ehrlich immune serum at the bottom.
FIG. 16.—Gel-diffusion test. Minced liver from C— mouse on left, Ehrlich tumour cells on
    right, absorbed anti-Ehrlich immune serum at the bottom

(2) *Gel-diffusion tests with the original immune sera*

The results obtained with the gel-diffusion test using the original immune sera and suspensions of normal and tumour cells homogenized, or lysed by the addition of merthiolate were parallel to those obtained in the *in vitro* tests. For equal quantities of material the order of reaction was . tumour more than whole blood, kidney, spleen, liver. The tumour homogenate or lysed suspension always gave a greater number of bands of greater strength than any other tissue tested, generally over 30 bands. Whole blood gave 20–25, kidney 15–20, spleen and liver between 10 and 15 bands.

(3) In vitro *tests with immune Sera adsorbed on normal tissues*
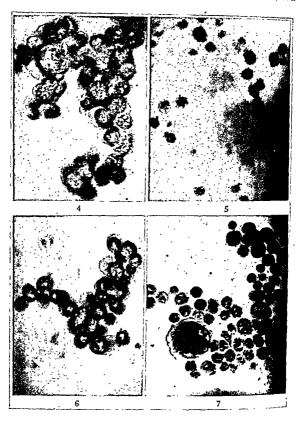
(a) *Absorption with whole blood of host mice.*—The addition of the blood or serum and erythrocytes of the host mice to the original immune serum resulted in the formation of a large quantity of precipitate. The precipitation was carried to exhaustion and the precipitate removed by centrifugation. The resulting antiserum was tested with normal and tumour cells *in vitro*. The antiserum was still strongly cytotoxic to tumour cells in the presence of added normal serum, inactive against erythrocytes, but still active against kidney and spleen cells, although it appeared to have hardly any effect on normal liver cells. Evidently some antibodies still remained in the antiserum which were active against kidney and spleen cells.

(b) *Absorption with liver, kidney and spleen.*—The antiserum which had been absorbed with blood was mixed with finely minced normal liver, kidney and spleen. Generally an equal volume of minced tissues was found to be sufficient. The resulting antiserum caused aggregation of the tumour cells but little lysis until fresh normal rabbit serum or guinea-pig serum was added, when aggregation became more conspicuous and cytolysis occurred. The tumour cells took longer to lyse than with the original immune serum. Instead of cytolysis being virtually complete within a few minutes it took anything from one-quarter to half-an-hour depending on conditions, although cell aggregation and degenerative changes in the cytoplasm were detectable almost immediately after the addition of the adsorbed immune serum (Fig. 4, 5). The antiserum showed little activity against the tumour cells at dilutions of greater than 20 times.
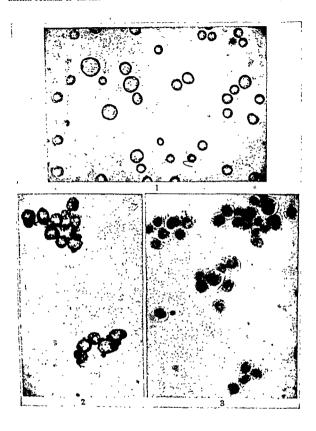
The normal kidney, spleen and liver cells from the host strain of mice were quite unaffected by incubation with either the absorbed immune serum alone, or with the absorbed immune serum with fresh normal serum added in that the cells survived as long as in normal serum alone and longer than in Locke's solution (Fig. 8–11).

Of particular interest was the observation that the absorbed Ehrlich ascites antiserum aggregated and lysed Landschütz ascites tumour cells though not so quickly under comparable conditions (Fig. 6, 7) Conversely, absorbed Landschütz ascites antiserum lysed Ehrlich ascites tumour cells. The two strains of tumour cells obviously had antigens in common not possessed by the normal cells of the host strains.
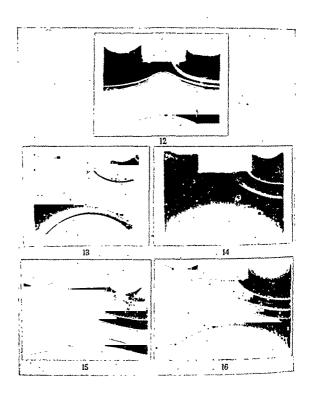
A sample of unabsorbed Ehrlich ascites antiserum which had been stored in a refrigerator at 4° for over 4 months was found not to produce lysis at the end of this period although it caused some aggregation of Ehrlich ascites tumour cells. The addition of fresh normal serum restored the cytotoxic activity although fresh normal serum alone had no harmful effect

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Easty and Ambrose.

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Easty and Ambrose.

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16

8   9   10   11

Easty and Ambrose.

It is very doubtful whether the antigens present in the cell membranes took part in the formation of any of the bands observed in the gel-diffusion tests since the quantity of material present in the membrane, which is approximately 50 Å thick, is minute compared with the quantity present in the remainder of the cell, even if it was capable of diffusion and contained only one antigen. It does not follow that the specific antibodies which give bands of precipitate with the diffusible antigens of the cells in the gel-diffusion tests are identical with those initiating cytolysis, since the process of cytolysis could not be stopped by washing the cells after the immune serum had been in contact with the cells for 5 minutes. This shows that the irreversible stage of cytolysis is reached very rapidly, and the most obvious cell component to be affected so quickly is the external cell membrane, especially as pinocytosis ceases almost immediately.

The degenerative changes which Schrek and Preston (1956) observed when immune serum acted on the Bagg lymphosarcoma cells were not the same as those observed in this work in all details, since they did not observe the swelling of the cytoplasm and formation of blisters which was characteristic of the cytolysis of the Landschütz and Ehrlich ascites cells by the immune serum. This formation of blisters was rarely observed during the cytolysis of normal cells by the immune serum. Kalfayan and Kidd (1953) observed similar cytoplasmic swelling with homologous immune serum. Likewise, Miller and Hsu (1956) observed very similar cytological changes caused by the action of rabbit and fowl antisera on the HeLa strain carcinoma cells. These latter workers state, however, that they found no evidence for tissue specificity, only for species specificity, whereas the results of both the *in vitro* and gel-diffusion tests with the absorbed immune serum demonstrated the presence of antigens in the tumour cells which were absent from the normal tissue cells of the host mice.

It is very unlikely that the specificity of the tumour cells, which has been demonstrated, is a cancer specificity although the gel-diffusion test showed the presence of at least three antigens in the tumour cells which were absent from the host's normal cells. These antigens are most probably associated with genetic differences between the host strain of mice and the Ehrlich and Landschütz strain of tumour cells, but it is of interest that such differences can be so readily detected using these techniques.

The presence of a soluble, diffusable antigen in Ehrlich ascites tumour cells which is absent from the Landschutz ascites tumour cells is particularly interesting in view of the fact that the Landschütz ascites tumours are almost certainly sublines of the Ehrlich ascites tumour (Tjio and Levan, 1954). The antigenic loss is quite large. After the absorption of the antibodies which are in common with the normal tissues of the host, the antigen responsible for the band in the gel-diffusion test with the Ehrlich ascites system which is absent in the Landschutz ascites system is one of the strongest, as judged by density of precipitate. It is obviously one of the components present in fairly high quantities. Whether it is cytoplasmic or nuclear in origin has not yet been determined.

SUMMARY

1. The immune sera to both the Ehrlich and Landschutz ascites tumours contained a number of antibodies against antigens present in the cells of the blood, liver, kidney and spleen of the host mice, indicating that these tissues had many, but not all, antigens in common.

reactions were sometimes obtained at the edge of the wells with blood and spleen preparations. These non-specific reactions were also obtained when blood and spleen preparations were allowed to diffuse into gelatin with no antiserum present, and were, therefore, the result of a reaction between substances present in the tissue preparations and the gelatin.

The results of the gel-diffusion tests were therefore in agreement with the result of the *in vitro* tests in that the absorbed immune Ehrlich antisera reacted with antigens present in the tumour cells but not with those present in the cells of the normal tissues tested.

DISCUSSION

All the immune antisera prepared contained high titres of antibodies to the antigens present in the normal tissues of the hosts. This result could not be explained entirely by the presence of contaminating normal cells of the host in the suspensions of tumour cells injected into the rabbits. The tumour cell suspensions injected were washed five or six times to remove any antigens present in the fluid medium, contamination with erythrocytes was very low after washing, and the ascitic fluid is supposed to represent a nearly pure culture of tumour cells. In spite of this between 80 and 90 per cent. of the precipitin reaction estimated either as the number of bands or quantity of precipitate present in the bands was caused by reaction of the antiserum with antigens found in normal tissues. It may be concluded that the Ehrlich and Landschütz ascites tumour cells have many antigens in common with the normal cells of the kidney, spleen and liver of the host mice, and particularly many in common with the erythrocytes of the host.

Nungester and Fisher (1954) found the presence of a mouse erythrocyte agglutinating antibody in rabbit antisera to mouse lymphosarcoma even when the antigenic material used for immunization appeared to be devoid of red blood cells on gross examination, and they suggested the possibility that the tumour cells and erythrocytes had antigens in common.

The cytotoxic action of the immune sera on the tumour cells was morphologically very similar to the action on normal cells, with the exception that the tumour cells showed a very characteristic formation of blisters of clear cytoplasm containing few granules. This was rarely observed with normal cells. Very similar effects have been observed when cells have been treated with compounds which are thought to combine with or change the nature of the cell membranes, e.g. compounds such as polyethyleneimine and anaesthetics. Schrek and Preston (1955) . . .

. . . from immune serum which had been heated at 56° for an hour to inactivate the complement. Similar results were obtained with a sample Ehrlich ascites antiserum in which the complement had probably become inactivated during prolonged storage, the tumour cells aggregated but cytolysis did not occur until fresh normal serum was added. Some antibodies were obviously combining with the cell membrane to produce aggregation although cytolysis would not commence in the absence of complement. Time lapse cinematography showed that there was a very rapid initial reaction between the immune sera and the cell involving the cell membrane.

A Cell Compressor for the Measurement of Mass and Concentration by Interference Microscopy

FOR a number of observations with the interference

example, Rousselet), but none provides quite the degree of control which is required. The compressor illustrated in Fig. 1 depends upon the bending of the slide S_2 and is therefore completely free from shear play and other uncontrollable movements,

with 'Araldite', avoids any disturbances due to changes in capillary attraction. The curvature of the meniscus at M is little affected by small changes in the gap G. These two conditions enable quite reversible changes in the gap to be effected over distances of about two microns. The actual thickness of the gap can be readily measured on the microscope with the help of an air bubble as previously described[1]. In addition, the medium surrounding the cells can be easily exchanged in this compressor without producing capillary disturbance.

Table 1   RESPONSE OF NORMAL LIVER AND HEPATOMA CELLS TO POLYETHYLENE-IMINE

Measurements of cytoplasmic concentration calculated from specific protein increment (ref. 2) and expressed as gm./100 c.c. water. Measured before and after introducing 0·12 per cent polyethylene-imine in calcium-free Locke's solution

Experiment 1*

Normal liver cells compressed to thickness of 8 5 × 10⁻⁴ cm.
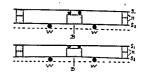
	Cell No.		
	1	2	3
Time	Concentration		
Zero, reagent introduced	10 8	11 7	11 7
7 min.	11 7	12 3	13 3
17 „	12 5	13 3	13 8
30 „	13 2	12 8	14 3

Experiment 2

Hepatoma cells compressed to thickness of 7 6 × 10⁻⁴ cm.

Time			
Zero, reagent introduced	16 7	16·3	17 2
10 min	16 7	0	21 4
20 „	11 2	0	22 7
30 „	0	0	16 3

The compressor can be used for direct observation with the interference microscope, for example, to immobilize ciliates such as *Paramecium* by light compression. It can also be used for measuring the refractive index and hence the concentration of material in various parts of the cell. It has been used, for example : (a) To study the effects of various positively charged polyelectrolytes upon normal and

decrease. (b) For measurements on *Amoeba proteus*. These indicate that the contractile vacuole has a concentration which is the same as that of the ... in the

the anterior end. Contraction cannot therefore be due to loss of water, as suggested by Pantin (1923)

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[1] Ambrose, E. J., *Proc. Roy. Soc.*, B (in the press)
[2] Barer, R., and Tkaczyk, S., *Nature*, 173, 821 (1954)
[3] Pantin, C. F. A., *J. Mar. Biol. Assoc. U.K.*, 13, 24 (1923)

2. The immune sera still contained antibodies to the tumour cells after absorption with the normal tissues of the host, as shown by *in vitro* and gel-diffusion tests.

3. Each absorbed serum was active against both types of tumour cell, producing lysis involving the formation of characteristic cytoplasmic blisters.

4. The immune serum, even before absorption, loses its cytotoxicity if the complement becomes inactive, although the tumour cells are still somewhat aggregated without lysis. Addition of fresh normal serum restores the cytotoxic activity.

5. The gel-diffusion tests with the absorbed immune serum show that there are at least three antigens in the Ehrlich ascites tumour cells, and at least two antigens in the Landschutz tumour cells which are not present in the normal cells of the kidney, spleen, liver and blood of the host.

6. The Ehrlich ascites tumour, of which the Landschutz tumour is almost certainly a subline, contains a major soluble diffusible antigen which is absent from the Landschütz tumour cells.

7. The specificity shown by the absorbed immune sera is not thought to be a cancer specificity, but 'due rather to genetic differences between the tumour strains and the host mice.

8. The techniques have already provided evidence for tissue specificity and it is hoped that they can be used for investigating the more subtle changes associated with induced tumours.

REFERENCES

HARRIS, M.—(1943) *J. exp. Zool.*, **93**, 131.
IMAGAWA, D. T., SYVERTON, J. T AND BITTNER   J. J.—(1954) *Cancer Res.*, **14**, 1
KALFAYAN, B. AND KIDD, J. G.—(1953) *J. exp Med*, **97**, 145.
KLEIN, G.—(1956) *Ann. N.Y. Acad. Sci*, **63**, 741.
LAMBERT, R A.—(1914) *J exp. Med.*, **19**, 277.
MACULLA, E. S.—(1948) *Yale J Biol. Med*, **20**, 343.
MILLER, D G. AND HSU, T. C —(1956) *Cancer Res.*, **16**, 306.
NUNGESTER, W. J. AND FISHER, H.—(1954) *Ibid*, **14**, 284.
OUCHTERLONY, O —(1948) *Acta Path. microbiol scand.*, **25**, 186
OUDIN, J —(1946) *Acad Sci*, *Paris*, **222**, 115.
PRESSMAN, D. AND KORNGOLD, L —(1953) *Cancer*, **6**, 619.
SCHREK, R. AND PRESTON, F. W.—(1956) *J. nat. Cancer Inst.*, **16**, 1021.
TJIO, J. H. AND LEVAN, A —(1954) *Acta Univ Lund.*, **50**, No. 15
VERNE, J. AND OBERLING, C.—(1932) *C R. Soc Biol Paris.*, **109**, 860.

Table I
Some totally synthesized natural products with biological activity

Fish liver oil
Vitamin A
and Carotenes

Me

$CH_2=CH-C=CH-CH=CH-C=CH$

Me   Me

Iater et al 1949

Animal Organisms

Nucleotides
e.g. ATP, FAD, etc.

Todd et al., etc. 1949-1954

Rauwolfia
Reserpine

MeO ... OCO ... OMe
MeO₂C ... OMe ... OMe

Woodward et al 1956

Scopola
Scopolamine

NMe
O.CO.CH.Ph
CH₂OH

Fodor et al. 1956

Nux vomica
Strychnine

Woodward et al. 1956

Ergot
Lysergic Acid

CO₂H
NMe
HN

Kornfeld et al and Woodward 1956

Endocrine Glands
Cortisone

C.CH₂OH
OH
O

Sarett, Reichstein et al. 1946

Opium
Morphine

NMe
OH   OH

Gates and Tschudi 1952

Pituitary
Oxytocin

PhOH   Et
NH₂   CH₂   CHMe
CH₂CHCONHCH₂CONH
S   CO
S   NH
CH₂CHNHCOCHNHCOCH(CH₂)₂CONH₂
CO   CH₂
CONH₂
CONHCHCONHCH₂CONH₂
CH₂
CHMe₂

Du Vigneaud et al 1952

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last thirty years or so with the synthesis of nearly all vitamins and some of the hormones and antibiotics is child's play. On the contrary, the most recent examples, as illustrated in Table I, represent great triumphs of synthetic chemistry. No—the difficulties I have in mind are connected with the planning, execution and timely cessation of research, dealing with simplification of structural elements of natural compounds or aiming at substances of novel structure and activity. This is where the man or woman who makes intelligent guesses, has imagination, a sense of discrimination and, at the same time, a knack of integrating all previous knowledge with new ideas will carry the day. My greatest admiration is for those who accept the frequent event of hitting a target B after having aimed at a target A, and who make the best of it.

Organization of research into therapeutic agents

There is one other facet the task of our friend must be greatly facilitated by the geographical proximity of chemical and biological experimentation, bringing about a most desirable cross-fertilization. Today, there exist a great number of well-known Institutes and Industrial Research Centres that have tried, each in their own way, to find an ideal answer to the question of how, under one roof, diversity can be combined with an all-pervading spirit of co-operation, and greatest possible liberty for the individual worker with productive integration. As you know there is no one answer, and it depends also on the group or groups of iatro-chemicals to be looked for and investigated by the research unit. The next Table (II) which I have used before on several occasions,[4] shows the main and sub-divisions of therapeutic agents: those, which later I shall use as illustrative examples, are encircled.

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been given in public by leaders in medical research such as Sir Henry Dale[1] and Sir Charles Dodds[2] and others, and only the year before last Dr. H. J. Barber discussed some aspects of it in his Chairman's Address to the Fine Chemicals Group on Chance and Design in the Search for new Drugs.[3]

The task of the iatro-chemist is made relatively easy if he deals with biologically active products occurring in nature. That does not mean that this sort of work which has reached its heights during the

THE STUDY OF BIOLOGICALLY ACTIVE AGENTS AS A VOCATION

JUBILEE MEMORIAL LECTURE 1956/57

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(Delivered at meetings of the following Sections of the Society of Chemical Industry: Yorkshire Section on October 15, 1956; London Section on November 12, 1956; South Wales Section on February 15, 1957)

FALSTAFF said to Prince Hal, " 'tis no sin for a man to labour in his vocation". Whatever his argument, you will agree his philosophy is sound because what the Oxford Dictionary describes as " a person's sense of being called to a task " should be in its execution a virtue and a blessing. The only difference between one case and the next lies in the way the call is received and followed. Some of us have the good fortune to hear it early and forcibly, and to obey it without hesitation and difficulties. Others, less fortunate, encounter obstacles and even experience frustrations in their vocational choice. If they are lucky they can go through a process of sublimation which, in this year after the Freud Centenary, should be generally known as a method for transforming anger into enthusiasm, dependency into devotion and despondency into productive effort.

Let us think of somebody among our friends who, for various reasons, is prevented from taking up the study of medicine; in spite of his very strong desire to practise one day the art of healing, circumstances: personal, economic, social, political, are against him. By some chance the career of chemistry and its related subjects becomes a practicable second choice. Commencing, perhaps, with feelings of regret and suppressed longing, our friend, overcoming this difficult period, sees more clearly every day the promise of a fulfilment previously not discernible. It is a slow process, this sublimation, but it is helped on by learning in organic chemistry of natural products and their use in the treatment of disease according to folklore and experience, and by the fascinating story of the successes of synthetic chemistry aiming at biologically active substances. There are the teachings of biochemistry, physiology and pharmacology which gradually open the doors to the understanding of the interaction between drugs and whole organisms, tissues or cells; there are the names of great men: Paracelsus, Pasteur, Ehrlich, Domagk, Dale, Fleming and others, and their work. Our friend, stimulated and aroused by all these new experiences, finds himself, with his labour and his thinking, engaged in what is called medicinal, pharmacological chemistry, or as reintroduced

he is to a smaller or larger extent contributing after all to the alleviation of the sufferings of man and beast, of course in difference to the practising

physician and surgeon, without being in contact with the patients under treatment. When he reaches the stage of fully enjoying his work and his results, he has, you will agree, successfully sublimated his original urge, and can now claim to participate in the study of biologically active agents as a vocation.

Three challenges

However, matters do not rest there; the iatro-chemist is continuously meeting a number of challenges to which he has to respond decisively. Working with or aiming at substances which, when applied to living organisms, produce certain effects, he has to draw on all the methods and accumulated knowledge of the various branches of chemical science, such as inorganic, organic, physical, bio- and pathological chemistry. This means that he must be either an ingenious " Jack of all Trades," a rare event, or that his personal activity must be integrated with those of a team of specialists. This is for him, and the others, challenge number one.

But even such an integrated team, meeting on the common ground of chemistry, will be unable by itself to produce new important biologically active compounds unless it enjoys the close collaboration of the biologist, the pharmacologist and, finally, the clinician. This is challenge number two, and it implies the acquisition by both sides of a common and unambiguous language, and on the part of the iatro-chemist of an understanding of the main principles of biology, physiology, bacteriology, experimental pathology and clinical medicine. It means that o~~ ~:~~~ L~~ :~ ~~~~ ~ ~ ~ :~ ~~ treble the re chemical colleague. Our friend has also, from time to time, to attend lectures, symposia and even congresses which deal with border-line subjects or subjects worked on by the biological camp. I am not trying to create a false sympathy for him but, as this represents an additional burden, he has to give up a great deal of his spare time. If he loves his job he will not complain. Maybe his family will !

The third challenge arises from a situation which, of course, is not unknown to exist in other fields of scientific activity, but which our friend, the iatro-chemist, recognizes as having very special features in his case: It concerns the problem of strategy and tactics of his kind of research and of the development work on any promising compound for final practical use. Consideration to these problems has

Similar principles apply, of course, to more complex organizations which embrace research activities such as those directed towards chemotherapy of microbial, protozoal and viral diseases, and which possibly cover the fields of hormones, nutrients, vitamins and vaccines. There exist so many examples here and abroad that there is no necessity to enumerate all the details and differences, some of which have been disclosed during the series of special lectures at the Royal Society, London,[5] on the Aims and Scope of Research Institutes. I prefer to give you instead an idealized picture of the lay-out of an Institute which deals, apart from matters of a fundamental nature, with the chemotherapy of malignant diseases, research with which I have been particularly connected for the last five years (Fig. 2). As compared with research organizations dedicated to other diseases or medical problems, the composition is complex and reflects the multiple and difficult approaches to the subject

research into biologically active agents (with which I have had and still have a few dealings myself), one pertaining to drugs effective in a certain manner on the central nervous system (CNS) and thus illustrating the pharmacodynamic group, and the other dealing with recent advances in cancer research. At first sight the two examples seem to have little in common. However, in course of my unfolding to you the second and third part of this lecture, I hope that you will, among other things, deduce on your own the potentialities of sedative and tranquilizing drugs as adjuvants in the treatment of an organic disease, such as malignancy in all its forms.

Psychopharmacological drugs

Of course there are other therapeutic uses for the agents assembled in Table III which have come into prominence during recent years, and for which the term " psychopharmacological " has been proposed. They are sub-divided into tran-
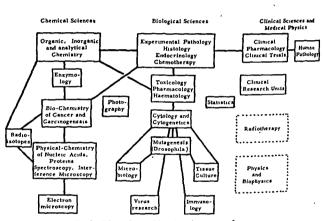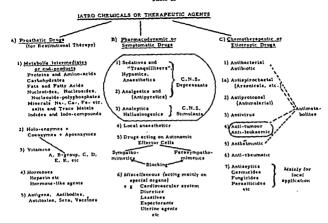
FIG 2.—Scheme of research into chemotherapy, etc, of cancer

of cancer. You notice that all the branches of chemistry and nearly all the disciplines of experimental biological sciences are represented. The proximity of a hospital is essential. While clinical research [illegible] problems are semi-independent, there exists an overall integration of effort for all applied matters, particularly with regard to attempts at chemotherapy.

I shall come back to some of the points touched upon just now when giving you two examples of

quillizers or ataractic drugs (ataraxia=peace of mind) and halluzinogenic or psychotomimetic agents, and represented by a number of substances of chemical heterogeneity, taking their origin from very old intoxicants or medicaments of natural sources. For mankind has, during all its history, a yearning for relief of pain and worries, a desire for a change of personality, however short-lived, for euphoria. The addiction of whole populations to opium, coca leaf chewing, cannabis resin eating and smoking and mezcaline consumption has led to a world-wide campaign and legislation against

Table II

IATRO CHEMICALS OR THERAPEUTIC AGENTS

A) Prosthetic Drugs (for Restitutional Therapy)

1) Metabolic intermediates or end-products
   - Proteins and Amino-acids
   - Carbohydrates
   - Fats and Fatty Acids
   - Nucleotides, Nucleosides, Nucleoside-polyphosphates
   - Minerals Na-, Ca-, Fe- etc. salts and Trace Metals
   - Iodides and Iodo-compounds

2) Holo-enzymes = Coenzymes + Apoenzymes

3) Vitamins A, B-group, C, D, E, K, etc

4) Hormones
   - Heparin etc
   - Hormone-like agents

5) Antigens, Antibodies, Antitoxins, Sera, Vaccines

B) Pharmacodynamic or Symptomatic Drugs

1) Sedatives and "Tranquillisers", Hypnotics, Anaesthetics } C.N.S. Depressants

2) Analgesics and (Antipyretics)

3) Analeptics Hallusinogenics } C.N.S. Stimulants

4) Local anaesthetic

5) Drugs acting on Autonomic Effector Cells
   - Sympatho-mimetics — Blocking — Parasympatho-mimetics

6) Miscellaneous (acting mainly on special organs) e g Cardiovascular system Diuretics Laxatives Expectorants Uterine agents etc

C) Chemotherapeutic or Etiotropic Drugs

1) Antibacterial Antibiotic

1a) Antispirochaetal (Arsenicals, etc.)

2) Antiprotozoal (Antimalarial)

3) Antivirus

4) Anti-tumour Anti-leukaemic

5) Anthelmintic

6) Anti-rheumatic

7) Antiseptics Germicides Fungicides Parasiticides etc } Mainly for local application

Antimetabolites

... be it academic, govern- ... ecialize in one of the ... pharmacodynamic or ... mbine work on two or ... en at the same place. Assuming that the principal activity consists of research into pharmacodynamic problems, then we might find in 1957 a lay-out as given in an idealized manner by Fig. 1. The dimensions of the squares are no fixed indications as to the true size of the sections or the personnel in them; that depends on special circumstances. For instance, industrial departments with interests in symptomatic drugs and, by necessity, orientated towards final development of *marketable products, are often dominated by the organic chemistry section.* I personally would say that the pharmacological and chemical sections should be roughly of an equal size and never geographically separated; the daily contact between the two divisions is too precious to suffer from distance.
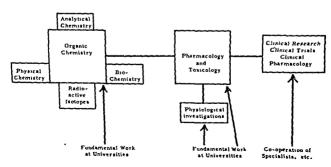
Fig 1.—*Scheme of research into pharmacodynamic drugs*

dangerous drugs (D.D.). But one of the oldest intoxicants not on the D.D. list, but resembling in some respects psychopharmacological agents, must be alcohol in all forms of fermented liquor.

Aldous Huxley, who in modern times has continued Baudelaire's literary study[6] into drugs creating artificial paradises,[7] expressed an opinion recently that due to inertia, the Westerner persists in using alcohol as his principal mind-transformer and anti-misery agent, and this in spite of all the progress made in the field of tranquillizing, euphoric and halluzinogenic compounds. Of course, the iatro-chemist and pharmacologist are not concerned with the creation of licit or illicit artificial paradises but solely with the right type of drug which, in the hands of the responsible physician or psychiatrist could be used to counteract excitement and anxiety in normal, neurotic and psychotic people, the latter suffering from one or the other kind of mental diseases, especially schizophrenia. This legitimate fight against worries, true and imagined, is in spite of what Huxley said, and of the existence of old plant remedies and their isolated and synthesized active principles, only at its beginning.

Table IV

TETRAHYDROCANNABINOLS
(Adams et al , Todd et al.)

R = C_5H_{11} isomer of Natural THC

= C_6H_{13} Synhexyl

= $CH.CH.C_5H_{11}$ RA 122
     Me Me

Cannabinol

Cannabidiol

All the drugs to be mentioned as psychopharmacological agents are acting in a depressant or stimulant

centres. This is not the place and the moment to discuss the physiology and pharmacology of these vital organs. Many problems are still unsolved and the enigma of the normal and pathological function,

and of their reaction to the effects of drugs, is only gradually puzzled out.[8]

One of the most pressing problems is the design of experimental conditions which will permit the assessment of psychopharmacological drugs. While the estimation of analgesic properties of morphine, its derivatives and synthetic analogues, is no mean task and this, and the testing for antagonism to motion sickness in the long run involves human "guinea-pigs," a technique for obtaining significant results as to the value of an ataractic drug has exercized and is exercizing the minds of many people. First, let me remind you that probably due to habit-forming properties no valuable psychopharmacological drug has so far been discovered in the analgesic group, although a piperidine derivative related somewhat to pethidine, such as Frenquel, has been found to counteract CNS disturbing reactions[9] and a morphine-scopolamine mixture has a long-standing reputation as a tranquillizer before major surgical operations. Scopolamine or hyoscine and synthetic compounds developed from atropine have formed the principal test substances for an investigation by Jacobsen et al.[10] Based on experiments by Pavlov and Masserman, he exposed cats and rats to conflict situations, such as frightening them away from an established mechanism of feeding or disturbing some other conditioned routine, which induced a kind of neurotic behaviour in the animals. After administration of scopolamine and benzilic acid ... ... alcohols to rats
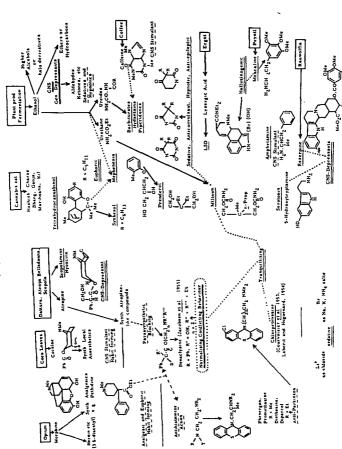
[illegible] effects were observed. ... ... pathetic blocking agents (see Ing's closely related Lachesine[11]) surprisingly possess an anti-psychic stress action.

On the other hand a substance derived from a family of anti-histamine and anti-Parkinsonism drugs, chlorpromazine, together with morphine, synthetic analgesics, barbiturates and ethanol, was strangely enough ineffective under Jacobsen's conditions. As chlorpromazine has been shown by different means to possess valuable depressant effects in certain mental diseases and psycho-neurotic states, this discrepancy brings out clearly the present difficulty with testing techniques, which include living beings as low as spiders[12] and fighting fishes[13] and as high as monkeys and man.

Further studies

But even studies on man can go wrong. This can be illustrated by the experience gained with a synthetic analogue of the active principles of Indian hemp resin, a very old euphoriant, peddled

Table III

Psychopharmacological drugs

Plant prod Fermentation

Coffee

Ergot

Preydil

Rauwolfia

Higher Alcohols or Ethers or halo derivatives

Ethanol

CNS Gen Depressants

Aldehydes Ketones, etc Sedatives and Hypnotics

Caffeine CNS Stimulant

Halluzinogenic

Mescaline

Ureides Hydantoins Piperidones

$NH_2.CO.NH$
COR

Urethane NH_2CO_2Et

Barbiturates Hydantoins Piperidones

Sedative, Anticonvulsant, Hypnotic, Anti-epileptic

Lysergic Acid

LSD

$H_2NCH_2CH_2$

Reserpine

CNS-Depressants

Cannabis sat Hashish Charas Bhang, Ganja, Marihuana, Kif

Tetrahydrocannabinol

$R = C_9H_{11}$

Euphoric

Mephesin

$R = C_6H_{13}$

Synhexyl

Miltown

Amphetamine CNS Stimulant $H_2N.CHCH_2$ Me

Serotonin 5-Hydroxytryptamine

Predecril

Datura, Atropa Belladonna Scopola

Scopolamine Hyoscine

Atropine

Synth Atropine-like compounds

CNS-Depressants

Coca Leaves

Cocaine

Synth Local Anaesthetics

CNS Stimulant Habit forming

Opium

Morphine

Heroin etc Synth (3,6-diacetyl)

Analgesics Pethidine

Synth Euphoric Habit forming

Antihistamine Action

Analgesic and Euphoric Habit forming

Parasympathetic Blocking

Benactyzine (Jacobsen et al. 1955)

Normalizing Conditiong Behaviour

Tranquillizing

Chlorpromazine (Courvoisier et al. 1953, Laborit and Huguenard, 1954)

$N(CH_2)_3CH_2$ NMe_2

Li^+ as chloride sedative

Br^- as Na, K, NH_4 salts

Phenergan Promethazine R = Me Diethazine Diparcol R = Et

Anti-Parkinson

$N CH_2.CH.CHNR_2$ Me

(445)

Table V

The three C's of the chemistry of cancer research

C1) Carcinogenesis   e.g   Nature of exogenous and endogenous carcinogenic agents
(cf. Miller and Miller; C. Heidelberger et al.)
Effects on Nucleic Acids ?   Interaction of such agents with 'Proteins (Miller, Haddow) and changes in or loss of proteins.

C2) Cell and Tissue Analysis (Normal, Abnormal and Embryonic)
Composition   Enzyme activity (content) and pattern (Greenstein and others, Everett and Martin).
Changes in cell surface properties (Ambrose, James and Lowick; Abercrombie et al.).   Trace metal contents (Bergel, Lewin).

C3) 'Chemotherapy'

Alkylating Agents

$-N(CH_2.CH_2.Cl)_2 = M$, $-CH-CH_2$, $-(CH_2)_3CO_2H$ H_2C-CH_2

Reaction with :

$-CO_2^-$

$>\!\!\overset{O}{\underset{\parallel}{P}}\!\!-O^-$

NH_2 or NH in pyrimidines, purines, histidine, sometimes α-amino groups.

e.g.
Chlorambucil

Amino-acid Derivatives
CB 1025 (Sarcolysine)

CB 3051

$MeSO_2.O-$
Myleran

$MeSO_2.O(CH_2)_4OSO_2Me$

CB 1506
$MeSO_2.OCH_2.CH_2Cl$

CB 1528
$MeSO_2.O.Et$

Antipurines, e.g. 6-mercaptopurine

Antimetabolites

Azaserine

DON

in the Orient under the names of hashish, charas, etc., and in America under the name of marihuana. When it was tried by Tayleur Stockings[14] in cases of neurotic and slight psychotic depression, he found it effective. But when Parker and Wrigley[15] included a control group in their clinical trial, no significant difference could be found between the placebo-receiving and the fully treated patients.

Chemically speaking, the basic structure of the material is that of tetrahydrocannabinol as given in Table III, occurring in nature in the form of double-number of analogues, particularly with different side-chains R, following the sequence of reactions as given on Table IV. Investigated pharmacologically by Macdonald[18] and Bain in this country and Loewe[19] in the U.S., these analogues showed various degrees of activity in the rabbit and in the dog. In addition to the trials on mental patients, synthetic tetrahydro-cannabinols were tested in epileptics with some success. So far no introduction into clinical medicine has been reported. This chapter, as mentioned before, well demonstrates the possibility of a negative practical outcome of concentrated work in many places, in spite of the fact that the natural precursor has gained such fame in the history of intoxicants and has given rise to the word "assassin."

Meanwhile, very much simpler phenol ethers were investigated by Berger and Bradley.[20] They demonstrated particularly with the o-tolylether of glycerol, mephenesin, that these ethers exerted an anti-convulsant action by depressing the lower centres of the central nervous system. Simplifying the molecular structure by discarding the phenol moiety, Berger and Ludwig[21] demonstrated a similar activity when using 2 : 2-diethylpropan-1 : 3-diol, Prenderol, with a gain of effectiveness on higher centres. This finding with an alcohol-derivative should take one back to ethanol with its general depressant properties, and its place as a kind of starting point in the development of general anaesthetics, ethers, halogen compounds, ureides and, with a glance at the CNS stimulant caffeine, to the barbiturates, hydantoins and piperi-dones as sedative hypnotics and ... epileptic ... form of in ... a recent date, the only effective drugs used by the psychiatrist, for quietening his patients, and in totalitarian countries by the police for extorting confessions. Combining the urethane with the Prenderol structure—notice the dialkylated C2 in comparison with the C5 in barbiturates, etc.—Ludwig et al.[22] arrived at the much talked about drug, meprobamate or Miltown, which is promoted, principally in America, as a remedy for relieving stress, reducing tension, irritability and restlessness and stabilizing emotion. What more could one wish for in a tranquillizing or ataractic agent?

There remain a number of drugs which are conveniently treated together because of the discovery of their interaction in the central nervous system, namely reserpine from Rauwolfia alkaloids and serotonine or 5-hydroxy-tryptamine (5HT) occurring in serum, intestines and brain, both also effective peripherally on blood pressure, and their counterparts

mental diseases and depressive states might be due to faulty metabolism of an adrenaline-like substance in the brain. Brodie et al.[23] proposed the idea that 5HT which raises blood pressure and acts in large doses as a CNS-depressant, was this neurohumural agent which subject to its formation from tryptophane and its destruction by amino-oxidase influenced the normal function of the brain. This hypothesis was supported by the observations that the tranquillizing drug reserpine was found to liberate 5HT from plasma and brain (excreted as 5-hydroxy-indolylacetic acid) and LSD, a compound producing profound mental disturbances in vitro to antagonize both the actions of the alkaloid and serotonine.

However, recent developments indicate that the true situation may be more complex.[24] Gaddum and Vogt[25] for instance, suggested among other things, that the antagonism between the halluzinogenic and ataractic agents might be unspecific and that other effects such as those on cerebral blood vessels ought to be taken into account. I cannot go into details but would like to mention that the 2-bromo-derivative of LSD also antagonizes the psychotomimetic action of LSD without showing definite suppression of the central effects of 5HT and without having an effect of its own. On the other hand the findings of Axelrod et al.[26] that the enzymic oxidation in vitro of LSD to 2-hydroxy LSD is inhibited to various degrees by chlorpromazine, serotonine and reserpine, may point to further, biochemical developments which will stimulate the synthesis of new compounds belonging to this most intriguing group of pharmacodynamic drugs, to which one must also count the inorganic ions Li and Br. It is hoped that these agents will never be misused either by the public or by any totalitarian authority.

It is necessary to leave now pharmacodynamic research and go on to my second example of research activities undertaken by the iatro-chemist, this time in one of the fields of chemotherapy.

Recent advances in cancer research

Being nearest my present interests, I am selecting that of cancer research. As compared with chemo-therapy ... the study ... being that of chemotherapy of viral diseases. In both cases chemists and biologists have to tackle not only extra- but mainly intra-cellular problems. Expressed in a simplified manner there are three C's of the chemistry (in the widest sense) of cancer research (Table V): Apart from chemotherapy of tumours

phosphorus or cellular constitutents) and residual blood, etc., results such as those obtained by my colleagues, Everett and Martin and myself[37] with rat livers should not be taken as absolute. But certain trends have become apparent when we compared the relative content in trace metals, participating in enzyme action (Cu, Mn, Mo and Zn) of livers of rats of different ages and from animals fed on butteryellow. We noticed, among other points, the relatively high copper content during the suckling period; a molybdenum content rising gradually from the foetal to the adult liver and being low in some butteryellow livers. The latter findings support some observations on the molybden... oxidase which is r... and as mentioned before, in tumour livers.[33]

One can imagine how these findings are being keenly followed up from the point of view of chemo-therapy, but for the greater part of research in this direction, as said before, one is still left with a *pedestrian empiricism*. Now, what is "news" on this front? In 1955 I reviewed,[4] at University College, London, and in Huddersfield, what are called the biological alkylating agents This subject and the related one of metabolic antagonists were taken up again in May, 1956, during a discussion at the Royal Society of Medicine by Davis, Galton and Haddow,[39] and others Most of the remedies in use at present belong to these two groups, but so far their most ... ...alignant ... ... :B 1348 ... ... mustard; ... ... diester; some ethyleneimine derivatives (e.g. TEM, TEPA), and anti-metabolites, such as 6-mercaptopurine. In addition hormonal substances, *stilboestrol*, *ACTH*, *cortisone*, are also employed, the oestrogenic compound giving some satisfaction in the treatment of prostatic carcinoma. A substance still under trial is the amino-acid derivative CB 3025 (studied independently from us[40] by Larionov et al[41] in Russia, as the racemate), and showing interesting differences in activities between the various optical forms, as found by Haddow.[42] The clinical evaluation is progressing slowly, first having given somewhat disappointing results, also encountered with an amino-acid derivative from moulds, L-azaserine and investigated in America[43] There it is now being followed by a synthetic analogue, 6-diazo-5-oxo-L-*nor*-leucine, DON.[44] Both these products have been shown to interfere with an enzymic step in the biosynthesis of inosinic acid[45] leading to other purines and then nucleic acids. Further studies of these two amino-acids, placed between the alkylating and anti-metabolic agents should give a better insight into the mode of action of amino-acid derivatives as anti-tumour agents.

Another advance, starting from the series of methanesulphonic acid esters was made by my colleagues, Haddow and Ross,[46] when they found ... ...anol ester CB 1506, was ... ... tumour, the other

Walker 200, ...

substances do, an effect on blood-elements. Some anti-tumour effect has been noted with the corresponding ethyl ester CB 1528. The latter compound was found to alkylate *in vivo* cysteine to S-ethyl cysteine, as demonstrated by Roberts and Warwick.[47]

Vigorous research is continuously going on, here, in Europe, Russia, and the U.S.A. There the large-scale screening of many products is magnificently carried out at the Sloan-Kettering Institute, New York, where Chester Stock et al.[48] have tested over 25,000 compounds and materials of natural origin. Our output at the Chester Beatty Research Institute is much more modest, and based on a somewhat different working philosophy, has through the study by Haddow of *ca*. 2000 compounds led to two drugs in use against certain forms of leukaemia and to several still under clinical test.

Finis

I hope I have given you in the relatively short time at my disposal a general and specific picture of how a person dedicated to the study of biologically active agents can make this a very worthwhile vocation. So many fundamental questions are still unanswered, so many practical problems need vigorous attacks; look around you and you find the physician, the patient and the so-far healthy waiting for small or large contributions to the understanding and treatment of virus-, cardiovascular, joint- and malignant diseases, to more knowledge of ageing.

May I end by quoting two great leaders in my own field who, though different in their ways, are thinking alike in the long run, A. Haddow and C. P. Rhoads. The latter finished an article on Cancer Quackery,[49] expressing something which is applicable to all research on therapeutic agents: "This termination will come only through hard, patient, orderly, co-operative study on an adequate but not excessive scale. It will be the result of multiple steady scientific steps of many investigators singly and in groups, toward the desired goal. And then fortunately we will find once more an endless frontier before us, to be further explored productively by devoted and *self-sacrificing* men and women, immune to adverse comment and concerned more with truth than with tradition." And A. Haddow[50] takes this further by saying that perhaps the greatest reward of a research career is its training and discipline, aspiring to the ultimate ideal in objectivity in the words of William the Silent, Prince of Orange: "It is not necessary to hope, to begin, nor to succeed, to continue" I ask you, could the student of biologically active agents following these words ever aim at a higher target?

References

1 Dale, An Autumn Gleaning, 1954. London, Pergamon Press
2 Dodds, *Chem. & Ind*, 1956, 650
3 Barber, *Chem. & Ind*, 1955, 1460
4 See Bergel, *J. Pharm*, Lond, 1955, 7, 297
5 Published in various parts of *Proc. roy. Soc.*
6 Baudelaire, "Les Paradis Artificiels," 1860 Poulet-Malassis, Paris
7 Huxley, A, "The doors of perception," 1954. Chatto & Windus, London

and leukaemias, C3, there remain the chemistry of carcinogenesis, C1, and cellular and tissue composition, C2. Logically speaking, if the riddles of C1 and C2 were solved, chemotherapy could be pursued as a matter of purely rational deduction. However, in spite of great efforts during the last few years, carcinogenic processes are only slowly elucidated, and knowledge of finer differences between cell or tissue composition of normal, abnormal and embryonic tissue, static and dynamic, is far from complete. In consequence, chemotherapy of cancer remains a field of empirical studies; it consists of a serious game of searching for a lead, and following it.

For some time attention has been given to the hypothesis[27] that carcinogenic action, by physical, such as radiation, or chemical means, by endogenous or exogenous agents, may lead in some instances to a loss, deletion or change of essential cellular

it is assumed, to a transformation of somato-genetic material in the nucleus and cytoplasm of the cell which is considered to be nucleo-protein in character. The next Table VI illustrates some of the evidence for the protein-change hypothesis: Miller and Miller[28] of America have noted interaction between liver proteins and 4-dimethylamino-azobenzene or butteryellow, which produces tumours in the liver on feeding. Recently Bhargava and Heidelberger[29] demonstrated that 1 : 2 : 5 : 6-dibenzanthracene, a carcinogenic polycyclic hydrocarbon, combined in form of an oxidation product, a

chemical point of view demonstrated that the liver of animals treated with carcinogenic azobenzene derivatives, like butteryellow, had in comparison with normal liver lost a proteinous antigen; while working at the Chester Beatty Research Institute he succeeded in tracking down a similar antigen loss in the kidney of male hamsters[31]; these had been treated, according to Kirkman by Horning,[32] with the oestrogen stilboestrol, causing the formation of tumours in that organ. The chemical and bio-chemical nature of the changed or deleted proteins has so far not been established, neither has the repercussion of such loss on the nucleic acids in the tumours or in the tumour-host been substantially elucidated.

But analytical studies on cellular constitutents have brought to light that the level of certain enzyme activities in tumours has fallen, although not to zero. For instance the liver of animals treated with butteryellow shows, according to Westerfeld et al.,[33] a lower level of xanthine oxidase activity. This molybdenoflavoprotein enzyme, the chemistry of which is studied at present by my colleague, Bray, others and myself,[34] catalyses the oxidation of hypoxanthine and xanthine to uric acid. Our American colleague I. Lewin investigated during the last two years in the Chester Beatty Research Institute the normal and abnormal mammary tissue of mice for the same enzymic activity and found[35] that it decreased per cell from the tissue of a low-tumour mouse strain via the non-tumorous tissue of a high-tumour strain (C+ mouse) to the tumour itself where it reached a minimum. Of course these observations do not allow for drawing a definite conclusion as to the xanthine oxidase being one of the proteins of diminishing returns. But at present one can accept the suggestion that something in the composition pattern of the malignant cell has changed. This, when submitted to further studies,[36] may one day help to explain fully the process of transformation from normal to malignant states.

Another analytical approach is the estimation of certain major and minor elements in parts of the

Table VI
Some carcinogenic agents which apparently interact with proteins

Butter yellow in the liver
(Miller and Miller)

1 2 5 6-Dibenzanthracene in the skin of the mouse
(C. Heidelberger, et al.)

Stilboestrol action on the kidney of the male hamster
(H. Kirkman, E. Horning, E. Weiler)

body by, say, emission spectroscopy or colorimetry. Because of inherent technical difficulties with biological material, particularly with regard to choice of base line (wet weight, dry weight, nitrogen,

Cyto-active Amino-acids and Peptides. Part III.[1,2] Synthesis of para-Substituted Phenyl and Alkoxymethyl Ethers of Tyrosine.

By F. Bergel *and* G. E. Lewis.

O-[*p*-Di-(2-chloroethyl)aminophenyl]-DL-tyrosine has been synthesised by two routes Etherification of *N*-acyl-L-tyrosine esters with *p*-fluoronitrobenzene led to racemisation Attempts to resolve *N*-acetyl-*O*-*p*-nitrophenyl-DL-tyrosine failed. Methoxymethyl and ethoxymethyl ethers of L-tyrosine have been prepared.

In Parts I[1] and II[2] of this series the preparation of novel *para*-substituted phenylalanines was described. One of them, *p*-di-(2-chloroethyl)amino-L-phenylalanine (CB 3025) was found to be very active against an experimental animal tumour,[3] to cause mutations in *Drosophila melanogaster*,[4] chromosome damage in tumour cells,[5] and inhibition of phenylalanine uptake in *Staph. aureus*.[6] In continuation of this work, syntheses of *para*-substituted phenyl and alkoxymethyl ethers of tyrosine were undertaken as a step towards the study of corresponding iodinated derivatives.

O-[*p*-Di-(2-chloroethyl)aminophenyl]-DL-tyrosine [II; R = (Cl·CH$_2$·CH$_2$)$_2$N] was obtained from diethyl acetamido-*p*-(*p'*-nitrophenoxy)benzylmalonate[7] (I; R = NO$_2$) *via* the *p'*-amino-compound[7] (I; R = NH$_2$), the preparative sequence being continued with hydroxyethylation, chlorination, and hydrolysis, in a manner published previously.[1] The reduction stage was improved and the di-(2-hydroxyethyl)amino-intermediate [I; R = (HO·CH$_2$·CH$_2$)$_2$N] was obtained crystalline. However, no attempt was made to isolate the malonate [I, R = (Cl·CH$_2$·CH$_2$)$_2$N], the chlorination product from the dialcohol [I; R = (HO·CH$_2$·CH$_2$)$_2$N] being hydrolysed directly to the required amino-acid [II; R = (Cl·CH$_2$·CH$_2$)$_2$N].

$$R\cdot C_6H_4\cdot O\cdot C_6H_4\cdot CH_2\cdot C(CO_2Et)_2 \qquad R\cdot C_6H_4\cdot O\cdot C_6H_4\cdot CH_2\cdot CH\cdot CO_2H$$
$$\underset{NHAc}{|} \qquad\qquad\qquad\qquad \underset{NH_2}{|}$$
$$(I) \qquad\qquad\qquad\qquad\qquad (II)$$

Attempts to synthesise the optically active forms of the tyrosine [II; R = (Cl·CH$_2$·CH$_2$)$_2$N], in particular the L-isomer, in view of the biological results in the phenylalanine series,[3] failed in every instance when *p*-fluoronitrobenzene was used with a number of *N*-acyl-L-tyrosine esters. Although etherification was achieved in reasonable yields, in presence of anhydrous potassium carbonate in ethyl methyl ketone, it was always accompanied by racemisation of the tyrosine ethers produced In consequence, only the DL-forms of *N*-formyl-, *N*-benzoyl-, and *N*-phthaloyl-*O*-(*p*-nitrophenyl)tyrosine ethyl esters were obtained. That this was due to the vigorous conditions necessary to effect etherification was shown by the results with fluoro-2·4-dinitrobenzene which, when condensed with *N*-formyl-L-tyrosine ethyl esters under milder conditions, gave the (2:4-dinitrophenyl)-*N*-formyl-*O*-L-tyrosine ester. We also confirmed the claim by Canzanelli *et al.*[8] that 3·4·5-tri-iodo-1-nitrobenzene, condensed with *N*-benzoyl-L-tyrosine ethyl ester, yields the L-form of di-iodinated *O*-*p*-nitrophenyltyrosine. *N*-Formyl-*O*-[*p*-nitrophenyl)-DL-tyrosine ethyl ester was reduced to the amino-compound, hydroxyethylated, chlorinated, and hydrolysed to the " mustard " derivative [II; R = (Cl·CH$_2$·CH$_2$)$_2$N], identical with the product from the malonic ester synthesis reported above. In the case of the *O*-*p*-nitrophenyl-*N*-phthaloyltyrosine ethyl ester, reduction gave the corresponding amine which, like its precursor, showed no optical activity.

... Feldberg, Abstract of Reviews, 1955, 20th Int. Physiol.

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19 L... ...
20 B
21 B
22 L
cf. als
23 E
1956, ...
24 cf. Gaddum, Abstr. of reviews, XXth Int. Physiol. Congr., 1956, 442
25 Gaddum & Vogt, Brit. J. Pharmacol., 1956, 11, 175
26 Axelrod et al., Nature, 1956, 178, 143; see also Rothlin, ibid., 1956, 178, 1400
27 Haddow, Ann. Rev. of Biochem., 1955, 24, 699
28 Miller & Miller, Adv in Cancer Res., 1953, 1, 339; Acad. Press, New York

29 Heidelberger & Bhargava, J Amer. chem. Soc., 1956, 78, 3671
30 Weiler, Z. f. Naturf., 1952, 7b, 324
31 Weiler, Brit. J. Cancer, 1956, 10, 553, 560
32 Horning and Whittick, Brit. J. Cancer, 1954, 8, 451
33 Westerfeld, et al., Cancer Res., 1956, 10, 486
34 cf. Avis, Bergel, Bray et al., J. chem. Soc., 1955, 1100; ibid., 1956, 1212, 1219
35 cf. Bergel, Bray, Haddow and Lewin, Ciba Symposium on the Chemistry and Biology of Purines, 1957, 256. J. & A.

... of Cancer. 2nd Ed., 1954,

... Vebb, in preparation

38 Greenstein et al., J. Nat Cancer Inst., 1943, 4, 271
39 Davis et al, Proc. R. Soc Med, 1956, 50, 1 ff.
40 Bergel et al, J. chem. Soc., 1954, 2409; ibid., 1955, 1223
41 Larionov et al., Lancet, 1955, ii, 169
42 cf B.E.C.C Ann Rep, 1953, 31, 6
43 Chester Stock et al, Nature, 1954, 173, 71
44 Dion et al., J Amer. chem. Soc, 1956, 78, 3075
45 cf. Buchanan et al., Ciba Symposium on the Chemistry and Biology of Purines, 1957, 249 J. & A. Churchill Ltd., London
46 Haddow & Ross, Nature, 1956, 177, 995
47 Roberts & Warwick, Nature, 1957, in press
48 Chester Stock, Current Res. in Cancer Chemotherap., 1955 3, 3
49 Rhoads, Cancer Res., 1956, 16, 373
50 Haddow, The Med. Press, 1952, 228, 247

(b) This material (11·8 g.) and *p*-fluoronitrobenzene (7·0 g.) in freshly dried, redistilled ethyl methyl ketone (200 ml.) were refluxed for 48 hr. over finely powdered anhydrous potassium carbonate (3·5 g.), and the solution was filtered, and taken to dryness *in vacuo* The residue was dissolved in ethyl acetate, and the solution washed with water, dried (Na_2SO_4), and evaporated to a small volume. After dilution with ether, addition of a small amount of pentane and storage in the refrigerator overnight, gave colourless N^α-*formyl-O-p-nitrophenyl*-DL-*tyrosine ethyl ester* (40%), m. p 105°, α 0° (Found : C, 60·4 ; H, 5·4; N, 7·8. $C_{18}H_{18}O_4N_2$ requires C, 60·3 : H, 5·4 ; N, 7·8%)

(c) The nitro-compound (4·78 g.) in ethyl acetate (100 ml) and methanol (25 ml) was. hydrogenated in the presence of palladised calcium carbonate (0 2 g). The filtered solution was' washed with water, dried (Na_2SO_4), and taken to dryness. N^α-Formyl-*O-p-aminophenyl*-DL-tyrosine ethyl ester was left as oil (yield 70%).

It gave an *acetyl derivative* which crystallised from aqueous ethanol as a monohydrate, m p. 85·5° (Found : C, 61 8 ; H, 6 3 , N, 7·1. $C_{20}H_{22}O_4N_2,H_2O$ requires C, 61·8 ; H, 6 2 ; N, 7·2%). The amine and its acetyl derivative were optically inactive.

(d) The amine was hydroxyethylated and chlorinated as for the malonic ester intermediate After acid hydrolysis the *O*-[*p*-di-(2-chloroethyl)aminophenyl]-DL-tyrosine was obtained, identical in m p and mixed m. p. with the product obtained by method (1).

O-p-Nitrophenyl-N-phthaloyl-DL-*tyrosine Ethyl Ester.*—(a) Equivalent amounts of L-tyrosine ethyl ester and phthalic anhydride were dissolved in benzene, the solvent was removed, and the reactants were heated as a thick oil on the water-bath for 30 min The product was refluxed in 2 5N-ethanolic hydrogen chloride for 1¼ hr., then evaporated to dryness *in vacuo*, and ethyl acetate added to the residual oil While the phthaloyl compound was dissolved by the ethyl acetate, unchanged amino-ester was deposited as the solid hydrochloride. The ethyl acetate solution was filtered, washed with aqueous sodium carbonate, and, after drying (Na_2SO_4), concentrated to a small volume Addition of pentane precipitated oily *N-phthaloyl-L-tyrosine ethyl ester* which solidified (yield 75%). A small portion was recrystallised from *cyclohexane*, giving white needles, m p 104—105°, $[α]_D^{20}$ −192° (*c* 2 01 in EtOH) (Found : C, 67·1; H, 5 2 ; N, 4·2. $C_{18}H_{17}O_4N$ requires C, 67·3, H, 5 1 ; N, 4 1%).

(b) This material was etherified by *p*-fluoronitrobenzene under the conditions used for the *N*-formyl compound After evaporation of the filtered ketonic solution, an oil (*ca.* 60%) was obtained which crystallised readily upon addition of ethanol Recrystallisation from ethanol gave O-*p*-nitrophenyl-N-phthaloyl-DL-tyrosine ethyl ester as colourless prisms, m. p. 116°, α 0° (Found : C, 65 1; H, 4 4, N, 6·1. $C_{25}H_{20}O_6N_2$ requires C, 65 2, H, 4 4 , N, 6·1%).

O-p-Aminophenyl-N^α-phthaloyl-DL-*tyrosine Ethyl Ester.*—The nitro-compound (15 g) in ethyl acetate (200 ml) and methanol (50 ml) was hydrogenated over palladised calcium carbonate (0 5 g) The filtered solution was shaken with water to remove methanol, separated, dried (Na_2SO_4), and taken to dryness The residual oil crystallised overnight (yield 80%). It recrystallised from propan-2-ol as light yellow needles, m p. 121—122°, α 0° (Found : C, 70 2 ; H, 5·1, N, 6 6. $C_{25}H_{22}O_5N_2$ requires C, 69 8, H, 5 2 ; N, 6 5%).

N-Benzoyl-O-p-nitrophenyl-DL-*tyrosine Ethyl Ester* —N-Benzoyl-L-tyrosine ethyl ester was prepared according to Canzanelli *et al* ,[8] it had m. p. 122—123°, $[α]_D^{20}$ −15·3° (*c* 3 0 in ethyl methyl ketone), $[α]_D^{20}$ −23 7° (*c* 3 7 in EtOH). It was treated with *p*-fluoronitrobenzene as above. The oil obtained after filtration and evaporation of the ketone crystallised in *ca.* 60% yield on addition of ethanol Recrystallisation from the same solvent gave *colourless needles*, m. p 145—146°, α 0° (Found : C, 66·5, H, 5 2, N, 6·1 $C_{24}H_{22}O_5N_2$ requires C, 66 4; H, 5 1; N, 6·5%)

N^α-*Formyl-O-(2 4-dinitrophenyl)*-L-*tyrosine Ethyl Ester* —N-Formyl-L-tyrosine ethyl ester in an exact equivalent of aqueous N-sodium hydroxide was heated *with an* equivalent amount of 1-fluoro-2 4-dinitrobenzene in ethanol on the water-bath for 30 min., water was added to precipitate the *dinitrophenyl tyrosine ether* (75%) The product solidified and, recrystallised from benzene-pentane, had m p 92—93°, $[α]_D^{15}$ +18° (*c* 1 8 in EtOH) (Found : C, 53 4 , H, 4·3; N, 10 5 $C_{18}H_{17}O_8N_3$ requires C, 53 6; H, 4 3 , N, 10·4%).

Attempts to Resolve N-Acetyl-O-p-nitrophenyl-DL-*tyrosine* —This compound, prepared by the method of Southwick *et al*,[7] had m p 162° (a) It was mixed with equimolecular proportions of brucine, cinchonine, cinchonidine, quinine, strychnine, or (+)-amphetamine in ethanol; *e g*, brucine (3 94 g) in ethanol (10 ml) was mixed with the tyrosine derivative (3 44 g) in ethanol (15 ml) No crystals were deposited during several days' storage at 20°. Evaporation

Our next efforts were towards the resolution of N-acetyl-O-p-nitrophenyl-DL-tyrosine by formation of salts with optically active bases such as brucine, quinine, cinchonine, cinchonidine, strychnine, and (+)-amphetamine. In no case were satisfactory results obtained. Equally unsuccessful was the application of the enzyme acylase I to the same tyrosine derivative under conditions similar to those used by Greenstein et al.[9] in the resolution of N-acetyl-DL-*alloisoleucine*.

In addition to the above phenyl ethers we prepared alkoxymethyl ethers of tyrosine, starting with N-phthaloyl-L-tyrosine ethyl ester. When this compound was allowed to react with chloromethyl methyl or ethyl ether and the phthaloyl group was removed with ethanolic hydrazine, O-methoxymethyl- and O-ethoxymethyl-L-tyrosine were obtained. Both products, in contrast to tyrosine, were easily soluble in water and liberated formaldehyde in warm dilute mineral acids. It was thought possible that this characteristic would enable these compounds to release formaldehyde slowly under biological conditions and that they would act as anti-tumour agents. However, no such effect was achieved with O-methoxymethyl-L-tyrosine (CB 3081) when applied by A. Haddow [10] to rats carrying the implanted Walker carcinoma 256.

Negative biological results were also obtained with the compound [II; R = (Cl·CH$_2$·CH$_2$)$_2$N] (CB 3051). This is surprising in view of the high activity of the p-di-(2-chloroethyl)aminophenylalanine.[3] On the other hand, Fahmy and Fahmy [11] observed point mutations with CB 3051 in *Drosophila melanogaster*.

<center>EXPERIMENTAL</center>

O-[p-Di-(2-chloroethyl)aminophenyl]-DL-*tyrosine* [II, R = (Cl·CH$_2$·CH$_2$)$_2$N] —*Method* (1)

(a) The nitro-compound (I, R = NO$_2$), prepared as described by Southwick et al.,[7] had m p 154° (lit, 153 5—154°), it (8·88 g) was hydrogenated in ethyl acetate (200 ml) and methanol (50 ml.) at atmospheric pressure in presence of palladised calcium carbonate (0·5 g) After filtration, the solvent was evaporated in vacuo and the residue dissolved in warm benzene (charcoal), which was filtered again. The cool solution on admixture with a small amount of light petroleum yielded, almost quantitatively, colourless crystals of the amine (I; R = NH$_2$) which, recrystallised from ethanol–light petroleum, had m. p. 150° (lit, m. p. 147—148°)

(b) Ethylene oxide (12 ml.) was added to a solution of the amino-compound (I, R = NH$_2$) (5 0 g.) in water (75 ml.) and glacial acetic acid (45 ml.). The mixture was kept at 20° for 24 hr, then neutralised by solid sodium hydrogen carbonate. The precipitated gum was extracted with ethyl acetate, and the solution dried (Na$_2$SO$_4$), treated with charcoal, and, after filtration, evaporated in vacuo to dryness. The residue (yield 65%), crystallised from benzene–light petroleum, gave the required di(hydroxyethyl)amino-compound [I; R = (HO·CH$_2$·CH$_2$)$_2$N] as colourless needles, m p 104—105° (Found · C, 62·3, H, 7 1, N, 5·7. C$_{24}$H$_{34}$O$_6$N$_2$ requires C, 62 2; H, 6·8, N, 5·6%).

(c) The foregoing compound (5 g.) was dissolved in sodium-dried benzene (200 ml) and ca 50 ml. of the solvent were distilled off, in order to remove any traces of water. To the cooled solution was added phosphorus oxychloride (15 ml), and the whole then heated under reflux for 25 min The benzene was removed in vacuo, and the residue dissolved in concentrated hydrochloric acid (120 ml), refluxed for 6 hr, and concentrated to a thick syrup in vacuo. This was dissolved in water (40 ml.), and the solution treated with charcoal, filtered, and neutralised by saturated sodium acetate solution, whereupon the O-[p-di-(2-chloroethyl)aminophenyl]-DL-*tyrosine* was deposited as a heavy off-white precipitate (35%). Recrystallised from aqueous methanol, it had m p 159—160° (decomp) (Found C, 57·2, H, 5·9; N, 7·0, Cl, 18·2 C$_{19}$H$_{22}$O$_3$N$_2$Cl$_2$ requires C, 57·4, H, 5 6, N, 7·1, Cl, 17 8%)

Method (2). (a) N-Formyltyrosine ethyl ester was mentioned by Haas et al [12] but no details were given In the present investigation it was prepared by treating L-tyrosine ethyl ester (5 g) with 98% formic acid (25 ml) and acetic anhydride (4 ml) at 40—50° for ½ hr The solvent was removed in vacuo and the residue diluted with water (250 ml) The aqueous solution was saturated with sodium chloride and extracted with ethyl acetate The extract was dried (Na$_2$SO$_4$) and concentrated to a small volume. Addition of ether precipitated N-formyl L-tyrosine ethyl ester (75%), m p 105—106° (lit, 105—106°), [x]$_D^{21}$ +37·2° (c 4 65 in EtOH) (Found · C, 60 5, H, 6 4, N, 5 9. Calc for C$_{12}$H$_{15}$O$_4$N · C, 60 8; H, 6 4, N, 5 9%).

Cyto-active Amino-acids and Peptides. Part IV.[1] Synthesis of α-[p-Di-(2-chloroethyl)amino-benzylidene- and -benzyl]-hippuramides and -hippuramido-acids.

By F. BERGEL and J. A. STOCK.

4-[p-D<small>L</small>-(2-chloroethyl)aminobenzylidene]-2-phenyloxazol-5-one [I; R = Ph; M = (Cl·CH₂·CH₂)₂N] is described. This compound, when treated with ammonia, primary amines, α-amino-acids, or α-amino-acid esters, gave "nitrogen mustard"-substituted unsaturated amides and peptides (as II and III) which were reduced to the corresponding substituted phenylalanine derivatives (as IV and V). An analogous thiazolone has also been prepared.

SYNTHESES of "nitrogen-mustard" analogues of L-, D-, and DL-tyrosine and of DL-thyronine were described in previous parts of this series,[1] which dealt with potential anti-tumour agents of an α-amino-acid character. We now report an extension of this work aiming at the preparation of related dipeptides.

Although known to be of limited value, Bergmann's azlactone synthesis[2] was employed because of the ready availability of p-di-(2-chloroethyl)aminobenzaldehyde[3] When the latter was heated ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯tate, the azlactone [I; ⋯ ⋯ ⋯ ⋯ ⋯ was obtained in yield⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯ ⋯nary amines to give α-[p-di-(2-chloroethyl)aminobenzylidene]hippuramides (II), and with amino-

acids and their esters to give the corresponding hippuramido-acids and -esters {III}. These compounds may be considered as dehydro-derivatives of Nα-benzoylphenylalanine amides and peptides. In order to achieve satisfactory reactions three or more molar equivalents of the amine or amino-acid derivative had to be used. The crystalline compounds prepared in this way are listed in Table I. They comprise derivatives of ammonia (II; R = H), ethylamine (II; R = Et), and cyclohexylamine (II; R = C₆H₁₁); of glycine (III; R = R' = H), DL- and L-alanine (III; R = Me, R' = H), L-valine (III; R = Me₂CH, R' = H); and of the ethyl esters of glycine (III; R = H, R' = Et), L-leucine (III; R = Me₂CH·CH₂, R' = Et), and DL-phenylalanine (III; R = Ph·CH₂, R' = Et). The azlactone (I; R = Ph) reacted with L-leucine, L-tyrosine ethyl ester, DL-aspartic acid, L-histidine, L-cysteine, and DL-methionine but the products resisted crystallisation. Aniline and p-aminobenzoic acid did not react with the azlactone {I; R = Ph) under the conditions outlined in the Experimental section, the azlactone being recovered unchanged.

Conversion of compounds of type (II) and (III) into DL-phenylalanine derivatives {IV) and (V) was first attempted by catalytic hydrogenation. This reduction of the glycine

(III; R = R' = H) and of the DL-alanine derivative (III; R = Me, R' = H) could be achieved only by using a mixture of Adams platinum oxide, palladium-charcoal, and Raney nickel. Any one or any pair of these catalysts was ineffective. However, it was found that the cyclohexylamide (II; R = C₆H₁₁) could be hydrogenated over Raney

gave a gum. When strychnine (3·34 g.) was used, the insolubility of the base required larger amounts of ethanol (75 ml.). In no case, including the use of methanol, chloroform, or aqueous ethanol (70%) as solvents, were satisfactory results obtained.

(b) The tyrosine derivative was treated in the manner described by Greenstein *et al.*[²] for acetyl-DL-*alloisoleucine*, in an aqueous solution of lithium hydroxide with dry acylase I powder. No preferential enzymic hydrolysis was observed.

O-*Methoxymethyl*-L-*tyrosine* —N-Phthaloyl-L-tyrosine ethyl ester (10 g.) and an excess of chloromethyl methyl ether (25 ml.) in dry benzene (200 ml.) were refluxed over dry potassium carbonate (5 g.) for 13 hr. The solution was filtered and evaporated *in vacuo*. The residual gum was extracted with a very large volume of boiling light petroleum (b. p. 60—80°) from which slowly crystallised, on cooling, O-*methoxymethyl-N-phthaloyl-L-tyrosine ethyl ester* (75%) as colourless needles, m p 75—76°, $[\alpha]_D^{20}$ −191° (*c* 3 01 in C_6H_6) (Found : C, 65·7, H, 5·75, N, 3 9 $C_{21}H_{21}O_4N$ requires C, 65 8; H, 5·5, N, 3·7%)

This compound was refluxed in an equivalent of M-ethanolic hydrazine for 2 hr., then cooled and filtered to remove phthalhydrazide, and the filtrate evaporated to dryness. The residue was passed in benzene down a column of alumina to remove any traces of phthalhydrazide. The eluate on evaporation gave an oil which was hydrolysed to the amino-acid by shaking it with a slight excess of N-sodium hydroxide at 20° for 2—3 hr. Most of the oil dissolved in the alkaline solution, which was then filtered and neutralised with an exact equivalent of N-acetic acid. The solution was then evaporated *in vacuo* at 40—50° until solid material was deposited A large excess of ethanol was then added to precipitate completely the O-*methoxymethyl*-L-*tyrosine* (yield 40%) Recrystallised from aqueous ethanol, it had m p. 185—190° (decomp) on slow heating, $[\alpha]_D^{23}$ −27·5° (*c* 2·00 in H_2O) (Found . C, 58·5, H, 6·9, N, 6·2 $C_{11}H_{15}O_4N$ requires C, 58 65, H, 6·7; N, 6 2%)

On warming a solution of the compound (0 25 g) in N-hydrochloric acid (5 ml) in a stoppered tube for 1—2 min, an odour of formaldehyde was produced Neutralisation with N-sodium hydroxide precipitated L-tyrosine The filtrate on addition of ethanolic dimedone deposited the formaldehyde derivative (m p and mixed m p)

O-*Ethoxymethyl*-L-*tyrosine* —In the same manner chloromethyl ethyl ether and *N*-phthaloyl-L-tyrosine ethyl ester gave O-*ethoxymethyl*-L-*tyrosine* (*ca* 10%) without crystallisation of the intermediate *N*-phthaloyl ethyl ester The compound, recrystallised from aqueous ethanol, had m p 188—190° (decomp), $[\alpha]_D^{23}$ −20·5° (*c*, 1·00 in H_2O) (Found C, 59 8, H, 7 0, N, 6 0 $C_{12}H_{17}O_4N$ requires C, 60 2, H, 7 2, N, 5·9%)

[1] Part I, Bergel and Stock, *J* , 1954, 2409
[2] Part II Bergel, Burnop, and Stock, *J* , 1955 1223
[3] Bergel, Haddow, and Stock, *B E C C Ann Report*, 1953, 31, 6
[4] Fahmy and Fahmy *J Genet* , 1956, 54, 146
[5] Koller and Veronesi, *Brit J Cancer* in the press
[6] Crathorn and Hunter, *Biochem J* in the press
[7] Southwick, Foltz and McIntyre *J Amer Chem Soc* , 1953 75, 5877
[8] Canzanelli Harrington and Randall *Biochem J* 1934 28 68
[9] Greenstein Birnbaum and Levintow *Biochem Preparations* 1953, 3 87
[10] Haddow personal communication
[11] Fahmy and Fahmy personal communication
[12] Haas, Suer and Landbourow *Biochim Biophys Acta*, 1951, 6, 1589

Table 1.

No	Compound	Isomer	Cryst. from	M.p.	$[\alpha]_D$	Yield (%)	Formula	Found (%) C	H	N	Cl	Required (%) C	H	N	Cl
1	II; R = H	—	A a	170—180°	—	95	—	—	—	—	—	—	—	—	—
2	II; R = Et	—	B b	165—166°	—	75	—	—	—	—	—	—	—	—	—
3	III; R = cyclohexyl	—	C c	177—178	—	76	—	—	—	—	—	—	—	—	—
4	III; R = R' = H	—	D	190—191	—	78	$C_9H_{10}O_4N_2Cl_4$	58·0	5·25	—	17·2	58·0	5·25	—	17·5
5	III; R = Me, R' = H	—	D	176—178	—	74	$C_{14}H_{18}O_4N_2Cl_4$	63·75	6·6	—	16·25	63·95	6·35	—	16·4
6	III; R = Me₂CH, R' = H	L	D	167—169	—	81	$C_{14}H_{18}O_4N_2Cl_4$	57·8	5·3	8·4	14·8	57·75	5·2	9·7	15·3
7	III; R = R' = Et	L	F d		+31° ± 1° e	82	$C_{14}H_{18}O_4N_2Cl_4$	57·2	5·7	8·9	14·7	57·75	5·2	8·6	15·3
8	III; R = Me₂CH, R' = Et	L	D	143—144	−15 ± 1·5°	79	$C_{15}H_{18}O_3N_2Cl_4$	59·2	8·3	8·0	14·75	59·3	5·5	8·8	14·0
9	III; R = Me₂CHCH₂, R' = Et	DL	A		+20 ± 2°	80	$C_{15}H_{18}O_2N_2Cl_4$	61·25	6·05	7·9	13·3	61·5	6·4	8·1	14·0
10	III; R = PhCH₂, R' = Et	DL	D e	141—142	—	73	$C_{19}H_{19}O_2N_2Cl_4$	64·0	5·6	7·55	12·15	63·9	5·7	7·2	12·2

A, Aqueous ethanol   B, Methanol   C, Ethanol   D, Aqueous Cellosolve   E, Aqueous propan-1-ol.   F, Pentanol.   a Colourless needles.   b Yellow prisms.   c c 1·64 in dioxan at 25°.   d Darkened somewhat on storage   e c 0·29 in EtOH at 25°.   f c 1·41 in dioxan at 24°.   g Yellow rods or needles.   h Calc. on azlactone which reacted; 50% recovered unchanged.

Table 2.

No	Compound	Isomer	Reduction	Cryst. from	M.p.	$[\alpha]_D$	Yield (%)	Formula	Found (%) C	H	N	Cl	Required (%) C	H	N	Cl
1	IV; R = H	—	A	D	171—172°	—	53	$C_9H_{12}O_2N_2Cl_2$	59·5	6·0	9·9	16·0	59·8	5·6	10·3	17·4
2	IV; R = Et	—	A	D	172—173°	—	71	$C_{14}H_{22}O_2N_2Cl_2$	59·0	6·4	10·3	16·0	58·8	5·6	10·3	17·4
3	V; R = R' = H	—	B	E	168—169°	0° ± 1° e	92	$C_9H_{12}O_2N_2Cl_2$	63·6	6·85	8·3	15·9	60·5	6·2	8·8	16·3
4	V; R = Me, R' = H	—	A	D	181°	—	—	$C_{14}H_{22}O_2N_2Cl_2$	56·7	5·3	8·6	16·0	58·0	6·7	9·0	—
5	V; R = Me, R' = H	DL	A	E	212—213·	—	65	—	—	—	—	—	—	—	—	—
6	V; R = Me, R' = H	L	B	D f	212—213·	0° ± 1°	84	$C_9H_{19}O_2N_2Cl_2$	57·0	5·55	8·7	14·6	57·5	5·6	8·75	14·8
7	V; R = Me₂CH·CH₂, R' = Et	L	A	E	186—188·	0 ± 1°	76	—	—	—	—	—	—	—	—	—
8	V; R = PhCH₂, R' = Et	DL	B	F	149—150	—	85	$C_{14}H_{22}O_2N_2Cl_2$	57·9	5·6	8·6	67·5	57·5	5·6	8·75	12·9
					152—153		57	$C_{19}H_{22}O_2N_2Cl_2$	61·8	6·0	—	61·1	6·2	6·7	—	12·2

The configuration given is that of the amino-acid attached to the N⁴-benzoyl-p,D-di-(2-chloroethyl)amino-DL-phenylalanyl group.   almost colourless.

G, A, Catalytic over Pt, Pd, and Ni.   B, By Zn-acid.   C, Catalytic over Raney Ni alone.   D, Methanol.   E, Aqueous methanol.   F, Aqueous ethanol.   a Rods or needles.   b Plates.   c Unchanged on admixture with preceding compound.   d Partial melting and resolidification, 212—213°.   e c 1·16 in EtOH at 21°.   f Shrinkage and softening at 102°, then resolidification.   g Not recorded.   h Prisms.   i Mixed m. p. with preceding compound, 212—213°.   c 0·79 in EtOH at 22°.   c 1·31 in dioxan at 22°.

nickel. Three other compounds were reduced, not always readily, with the three-catalyst mixture, namely, the derivatives of ammonia (II; R = H), L-alanine (III; R = Me, R' = H), and DL-phenylalanine ethyl ester (III; R = Ph·CH₂, R' = Et).

While zinc in glacial acetic acid was not effective, the addition of concentrated hydro-chloric acid to this system led to very rapid and efficient reduction, yields being substantially higher than by catalytic hydrogenation (Table 2). The apparent lack of optical activity in the reduced L-alanine and L-leucine ethyl ester derivatives (Table 2; Nos. 6 and 7), the former prepared by either method of reduction, does not indicate that racemisation had occurred. After hydrolysis of the compounds with 6N-hydrochloric acid, the solutions showed rotations comparable with those to be expected from the presence of an equivalent concentration of L-alanine and L-leucine respectively. Moreover, the m. p. of the L-alanine derivative (Table 2, No. 6) was about 25° lower than that of the corresponding DL-alanine peptide (No. 5).

The methods outlined above thus enabled us to prepare several Nᵅ-benzoyl-dipeptides carrying a "nitrogen mustard" group on the phenylalanine moiety. The benzoyl group does not, however, lend itself to removal by means which would preserve the peptide link. We therefore sought to vary the azlactone so as to have ultimately an easily removable N-acyl group. To this end we attempted to prepare the azlactones (I; R = Me), (I; R = CF₃), (I; R = Ph·CH₂), and (I; R = Ph·CH₂·O) but failed. We did, however, synthesise the thiazolone (VI) from phenyl(thioacetyl)glycine but this did not give crystalline products when allowed to react with ethylamine or cyclohexylamine. This approach was therefore postponed. However, as indicated in a preliminary communication,[4] we are investigating the use of the readily removable o-cyanobenzoyl group in the preparation of peptides related to the compounds described in this paper but possessing a free amino-group. That such an amino-group may be required for powerful anti-tumour activity is illustrated by the apparent ineffectiveness[5] on the Walker carcinoma 256 of the azlactone (I, R = Ph), the thiazolone (VI), of the dehydro-compounds as given on Table 1, and of the glycine peptide (V; R = R' = H) While none of these compounds has been tested yet up to the limit of tolerance, it seems that pronounced anti-tumour action in this series may depend on the presence of zwitterion systems.

4-[p-Di-(2-chloroethyl)aminobenzylidene]-2-phenyloxazol-5-one (I; R = Ph) (cf. Buck and Ide[6]).—*p*-Di-(2-chloroethyl)aminobenzaldehyde[3] (4·92 g.), hippuric acid (3·58 g., 1·0 mol.), powdered anhydrous sodium acetate (1·64 g., 1·0 mol.), and acetic anhydride (6 ml.) were heated with stirring at about 120° Complete dissolution occurred within a few minutes The red solution was then heated on a steam-bath for about 25 min It deposited red crystals on cooling. Water (7 ml) was added to the cold mixture. The aqueous layer was poured off after about 15 min, and the solid residue washed with water, collected, and dried The *oxazolone* crystallised from benzene in bright red prisms (4·89 g, 63%), m. p. 132—135° (with previous softening, and clarification at 142°) Omission of sodium acetate from the reaction mixture resulted in a slightly lower yield Recrystallisation from benzene raised the m p to 135—138° (softening from 122°, clarification and meniscus 142—145°) (Found C, 64·0; H, 6·0; N, 7·0; Cl, 16·5 C₂₀H₁₈O₂N₂Cl₂·¼C₆H₆ requires C, 64·5, H, 4·9, N, 6·5; Cl, 16·6%). Crystallisation from Cellosolve gave light tan solvent-free prisms of much sharper m p (141—142°) (Found. C, 61·5, H, 4·7, N, 7·4, Cl, 18·0 C₂₀H₁₈O₂N₂Cl₂ requires C, 61·7; H, 4·6, N, 7·2; Cl, 18·3%). Moistening these with a little benzene caused the colour of the compound to revert to bright red. Both solid forms showed strong fluorescence under ultraviolet light.

2-Benzyl-4-[p-di-(2-chloroethyl)aminobenzylidene]thiazol-5-one (VI) (cf Abraham *et al.*[7]).— Phenyl(thioacetyl)glycine[8] (2·09 g.), *p*-di-(2-chloroethyl)aminobenzaldehyde[3] (2·46 g., 1·0 mol.), and acetic anhydride (5 ml) were shaken with gentle warming until dissolution was complete, then kept at 50—60° for 3 hr. After cooling, a solid was deposited; this was collected and washed with ethanol, then light petroleum The orange rods (2·09 g, 50%), m. p 153—155°, were recrystallised from benzene-ethanol, then Cellosolve, and yielded the *thiazolone* (1·6 g, 38%), m. p. 157—158° (Found C, 60·2; H, 5·1; N, 6·5, S, 7·3, Cl, 16·8. C₂₁H₁₈ON₂SCl₂ requires

[1] Part I, Bergel and Stock, *J.*, 1954, 2409; Part II, Bergel, Burnop, and Stock, *J.*, 1955, 1223; Part III, Bergel and Lewis, *J.*, 1957, 1816.

[2] Carter, "Organic Reactions," Wiley, New York, 1946, Vol. III, p 198

[3] Anker and Cook, *J.*, 1944, 489

[4] Bergel and Stock, *Proc Chem Soc.*, 1957, 60.

[5] Personal communication from Professor A Haddow.

[6] Buck and Ide, *Org. Synth*, Coll Vol. II, 1943, p 55.

[7] Abraham, Baker, Chain, and Robinson, "Chemistry of Penicillin," ed. Clarke, Princeton Univ. Press, 1949, p. 848

[8] *Idem, op cit*, p. 778

[9] Cf Greenstein, Birnbaum, and Otey, *J. Biol Chem*, 1952, 204, 207.

C, 60·15; H, 4·8; N, 6·7; S, 7·6; Cl, 16·9%). The use of anhydrous sodium acetate and of a higher reaction temperature offered no advantage.

α-[p'-Di-(2-chloroethyl)aminobenzylidene]hippuramides (II).—A concentrated aqueous solution of ammonia or of diethylamine (±3 mol. equiv.) was added to an acetone solution containing the oxazolone (I; R = Ph) (0 5—1·0 g. per 10 ml.). The ammonia reaction was carried out at room temperature and was complete within 15 min. The ethylamine reaction mixture was heated under reflux for a few minutes. Reaction with cyclohexylamine was carried out by heating under reflux a solution of the oxazolone (I, R = Ph) and base (3 mol. equiv.) in ethyl acetate for ca. 1 hr. The fading of the initial orange colour of the solution to lemon-yellow was a guide to the rate of reaction in all cases. The mixtures were evaporated to dryness under reduced pressure and the amides were crystallised from suitable solvents (Table 1)

α-[p-Di-(2-chloroethyl)aminobenzylidene]hippuramido-acids and -amido-esters (III; R' = H or Et).—(a) Reaction of oxazolone (I; R = Ph) with amino-acids. The oxazolone was dissolved in boiling acetone (ca. 10 ml. per mmol. of oxazolone) and treated with a solution of the amino-acid (3 mol.) in N-sodium hydroxide (3 equiv.). The mixture was heated under reflux for 15 min., by which time the colour had usually faded to pale yellow. Most of the acetone was evaporated under reduced pressure, the residual aqueous solution diluted with a few ml of water, and the product precipitated by addition of dilute hydrochloric acid to pH ca. 3. The products which could be purified by crystallisation are tabulated in Table I.

(b) Reaction of oxazolone (I, R = Ph) with amino-acid ethyl esters. The oxazolone was refluxed for up to 1 hr. in ethyl acetate with the amino-ester (5 mol ; usually prepared from the ester hydrochloride by treatment with triethylamine in chloroform). The solution was evaporated, and the residue washed with dilute (ca. 0·1N-)hydrochloric acid to remove excess of amino-ester. Products obtained crystalline are given in Table 1

α-[p-Di-(2-chloroethyl)aminobenzyl]-hippuramides and -hippuramido-acids and -esters (IV and V).—(a) Catalytic reduction. The unsaturated compound was shaken in methanol or methanol–ethyl acetate over Adams platinum oxide catalyst, Raney nickel, and 5% palladium–charcoal The cyclohexylamide (II; R = C₆H₁₁) was successfully reduced in the presence of Raney nickel alone. The results are set down in Table 2.

(b) Chemical reduction. The unsaturated compound (1 mmol.) was dissolved in glacial acetic acid (10 ml.), and concentrated hydrochloric acid (3 ml.) was added Zinc dust (2 g.) was then added in two or three portions to the stirred and cooled solution Stirring was continued for 10 min., though the colour of the solution generally disappeared within about 3 min. The mixture was filtered, the residual zinc washed with a little acetic acid, and the combined filtrate and washings were diluted with water to about 30 or 40 ml. The solution was brought to pH ca. 3 by the addition of N-sodium hydroxide, and the precipitated product was crystallised. The results are given in Table 2.

Hydrolysis of Reduced Peptides of L-Alanine and of L-Leucine Ethyl Ester—(a) L-Alanine derivative (V; R = Me, R' = H). The compound (101 mg.) was heated for 3 hr under reflux in 6N-hydrochloric acid (2 ml.). Colourless needles, presumably benzoic acid, collected in the

above in which the reflux time of the compound (50 mg.) in 6N-hydrochloric acid (1·5 ml.) was 4 hr. gave a solution (final vol., 1 5 ml.) whose α²⁵_D was +0 09° ± 0 015° Hence, for the leucine released (11·9 mg., assuming complete hydrolysis), [α]²⁰_D = +11·5° ± 2° (c 0·79 in 6N-HCl). The L-leucine used had [α]²⁵_D +16° ± 2° (c 0·84 in 6N-HCl).

stromverteilung[12], als Trennmethode für Flavine zu erproben. A. EMMERIE[13] fand,
daß sich Riboflavin von FMN durch Verteilung zwischen Wasser und Benzylalkohol
trennen läßt, und A. O. BESSEY[14] gelang anschließend eine teilwese Trennung von
FMN und FAD mit dem gleichen Lösungsmittel, das auch von K. YAGI[15] benutzt
wurde. YAGI zog überdies bereits die Gegenstromverteilung zur Untersuchung von
Flavinen heran. Er bediente sich hierbei eines Lösungsmittel-Systems aus Butanol,
Essigsäure und Wasser; allerdings gelang damit nur eine partielle Trennung von Ribo-
flavin, FMN und FAD[16].

Die Verwendung von Phenol (oder Kresol) bei der Gegenstromverteilung von
Flavinen war naheliegend, da dieses einerseits von O. WARBURG und W. CHRISTIAN[17]
bei den Extraktionsverfahren, die zur Isolierung von natürlichem FAD geführt hatten,
benutzt worden war, und da andererseits G. H. HOGEBOOM und G. T. BARRY[18] das
System Phenol-Äther/wäßr. Kaliumchlorid erfolgreich zur Gegenstromverteilung von
Diphosphopyridinnucleotid herangezogen hatten.

Das von uns gewählte Lösungsmittelsystem besteht aus einer unteren organischen
Phase von Phenol und Kresol (2:1) und einer oberen wäßrigen Phase aus $0.054 m$
Phosphatpuffer vom p_H 7.4. In der nachstehenden Tabelle sind die Verteilungs-
quotienten K verschiedener Flavine in diesem System aufgeführt.

Verteilungsquotienten

Flavin	Riboflavin	FAD	FMN	Riboflavin-„Polyphosphat"
K	< 0.05	0.4–0.5	1.3–1.9	> 10

Im Falle von FMN haben wir gefunden, daß K zu Konzentrationsänderungen des
Flavins im Bereich von $8 \cdot 10^{-3} m$ bis $2 \cdot 10^{-5} m$ nicht sehr berührt wird; bei unseren
eigentlichen Gegenstromverteilungen wandten wir noch höhere Konzentrationen an,
ohne daß sich ein nachteiliger Effekt auf die Wirksamkeit der Trennmethode bemerk-
bar machte. In einigen Versuchen mit einem Lösungsmittelsystem aus Kresol als
alleiniger organischer Phase und einem Puffer von niedrigerem p_H ergab sich, daß der
Verteilungsquotient K von FMN im oben erwähnten Bereich mit wachsender Kon-
zentration rasch anstieg. Dieser Unterschied zwischen den Systemen hängt vermut-
lich mit der geringeren Löslichkeit von Riboflavin-Derivaten in Kresol als in Phenol
zusammen.

S.   ... of Organic Chemistry, Vol. III,

                                            ), 755 (1949)
                                            [Tokyo] 43, 93 (1956)
[C. A. 50, 11415 (1956)].
[16] K. YAGI und J. OKUDA, Vitamins (Japan) 7, 991 (1954).
[17] O. WARBURG und W. CHRISTIAN, Biochem. Z. 298, 150 (1938).
[18] G. H. HOGEBOOM und G. T. BARRY, J. biol. Chemistry 176, 935 (1948).

DIE TRENNUNG VON RIBOFLAVIN-DERIVATEN DURCH GEGENSTROMVERTEILUNG

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Für die Trennung von Flavinen durch Gegenstromverteilung wird ein Lösungs-
mittelsystem angegeben, das aus Phenol, Kresol und Phosphatpuffer besteht.
Die beschriebene Methodik läßt sich zur Reinigung von FMN des Handels, zur
Trennung von FAD und FMN sowie zur Isolierung und Charakterisierung
von FAD aus Rinder-Xanthinoxydase anwenden

Zur Trennung von Gemischen von Lactoflavin (Riboflavin), Lactoflavin-5′-phos-
phorsäure (Flavin-mono-nucleotid, FMN), Flavin-adenin-dinucleotid (FAD) und
verwandten Flavinen***) sind bereits mehrere Methoden beschrieben. Die meistge-
brauchte Technik war bisher die Chromatographie: zur Säulenchromatographie
dienten Tonerde[1], Florisil[2], Calciumphosphat[2], Cellulose[3,4] und Ionenaustauscher[5];
für die papierchromatographische Trennung[2,6,7] wurden verschiedene Lösungsmittel-
systeme angegeben. Auch die Elektrophorese fand, sowohl in Versuchen an Cellulose-
säulen[8] wie auch in Form der Papierelektrophorese kleineren Maßstabs[8,9,10], An-
wendung auf das vorliegende Problem.

Obwohl einige der genannten Verfahren präparativ recht brauchbar sind, erschien es
doch einladend, die zumal in ihrer vollautomatischen Ausführung[11] bequeme Gegen-

[1] K. BURTON, Biochem. J. **48**, 458 (1951).
[2] E. DIMANT, D. R. SANADI, und F. M. HUENNEKENS, J. Amer. chem. Soc **74**, 5440 (1952).
[3] ⸏ ᴳ ⸍⸍ ⸍ ⸍ ⸍ ⸍ ⸍ ⸍ ⸍⸍⸍⸍ ⸍ ⸍⸍⸍ ⸍ ᵟ⸍⸍
[4] ⸏ ⸍⸍ ⸍ ⸍ ⸍ ⸍ ⸍ ⸍ ⸍ 46.
[5] ⸍ ⸍ ⸍ ⸍⸍ ⸍ ⸍⸍ ⸍ ⸍ ⸍ ⸍ ⸍ ⸍ ⸍ ⸍
[6] ⸍ ⸍⸍⸍⸍ ⸍ ⸍ ⸍ ⸍ ⸍⸍
[7] F. M HUENNEKENS, D. R. SANADI, E. DIMANT und A. J. SCHEPARTZ, J. Amer. chem. Soc.
75, 3611 (1953).
[8] P. CERLETTI und N. SILIPRANDI, Biochem J **61**, 324 (1955).
[9] J. L. PEEL, Biochem. J. **58**, XXX (1954).
[10] K. YAGI und Y. MATSUOKA, J. Biochemistry [Tokyo] **42**, 757 (1955) [C.A. **50**, 6557
(1956)].
[11] L. C. CRAIG, W HAUSMANN, E H. AHRENS JR. und E. J HARFENIST, Analytic. Chem **23**,
1236 (1951) — Unser Apparat wurde von A. R. GILSON und A. WRIGHT konstruiert.

HII

Flavine, erregten unser Interesse und veranlaßten uns, die Gegenstromverteilungs-
methode zur Untersuchung der Xanthinoxydase der Kuhmilch heranzuziehen. Es
sollte weitere Klarheit darüber erbracht werden, inwieweit das Flavin in'der über-
stehenden Lösung Hitze-denaturierter Xanthinoxydase mit FAD identisch ist.
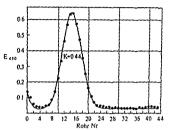
Abbildung 3. Gegenstromverteilung von FAD aus Xanthinoxydase (43 Überführungen)

Die zu dieser Untersuchung benötigte Xanthinoxydase wurde nach der Vor-
schrift von P. C. AVIS, F. BERGEL und R. C. BRAY[21] dargestellt. Abbildung 3 zeigt
eine Verteilung des daraus erhaltenen Flavins. Das Hauptmaximum ($K = 0.44$) weist
keine Zeichen von Inhomogenität auf. Man erkennt lediglich eine kleine Substanz-
menge von niedrigem Verteilungsquotienten — vermutlich Photolyseprodukte —
aber keine sonstigen Maxima. Diese Feststellung, zusammen mit Ergebnissen der
Papierchromatographie und den Absorptionsmessungen (vgl. Versuchsteil), stützen die
Vorstellung[20], daß die Flavin-Komponente der Xanthinoxydase FAD ist.
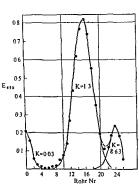
BESCHREIBUNG DER VERSUCHE

Allgemeines

Zur Herstellung des Losungsmittel-Systems diente Phenol (laboratoriumsüblicher Reinheits-
grad), das durch Zugabe von Wasser ($1/10$ des Gewichtes) und Erwärmen verflüssigt worden
war; das verwendete Kresol (Isomerengemisch) war B P.-Ware Bei der Isolierung des
Flavins aus Xanthinoxydase benutzten wir reinstes Phenol (AnlaR) und redestilliertes Kresol. —
$1/$ Wasser wurde mit $0.29/$ Kresol, $0.58/$ Phenol, 1.18 g $NaH_2PO_4 \cdot 2H_2O$ und 6.16 g Na_2HPO_4

21) P. G. AVIS, F. BERGEL und R. C. BRAY, J chem. Soc. [London] 1955, 1100.

Die mit einem *FMN-Handelspräparat* erhaltene Verteilungskurve zeigt Abbildung 1. Man erkennt ein Hauptmaximum, das dem FMN (K = 1.3) und 80.4% der bei 450 mµ absorbierenden Substanz entspricht; die zwei weiteren Maxima repräsentieren Substanzmengen von 5.4% (K = 0.03) und 14.2% (K = 8.6). Vermutlich ist die erste Begleitsubstanz Riboflavin (obgleich Photolyseprodukte gleichfalls niedrige Verteilungsquotienten besitzen), die zweite ein Riboflavin, das mehr als eine Phosphatgruppe trägt. Eine auf diesem Ergebnis aufgebaute präparative Reinigung von FMN ist im Versuchsteil beschrieben.
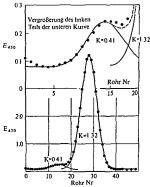
Abbildung 1 Gegenstromverteilung eines FMN-Handelspräparates (26 Überführungen)

Abbildung 2. Gegenstromverteilung eines FMN-FAD-Gemisches (49 Überführungen)

Die *Abtrennung von FAD*, synthetisch erhalten nach S. M. H. CHRISTIE und Mitarbb.[4] aus einem Gemisch mit einem Überschuß von *FMN*, geschah durch 2 aufeinanderfolgende Verteilungsoperationen: die erste bewirkte eine partielle Trennung; die zweite wird durch Abbildung 2 veranschaulicht.

Untersuchung des Flavins aus Xanthinoxydase. Der Befund von T. P. SINGER und E. B. KEARNEY[19], daß gewisse FAD-Peptide im D-Aminosäureoxydase-Test, der bisher als streng spezifisch für FAD selbst galt[20], aktiv sind, sowie Arbeiten von F. M. HUENNEKENS und Mitarbb.[7] über nahe verwandte, aber mit FAD nicht identische

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[19] E. B. KEARNEY, V. MASSEY und T. P. SINGER, Federat. Proc. 15, 286 (1956)
[20] H. S. CORRAN, J. G. DEWAN, A. H. GORDON und D. E. GREEN, Biochem. J. 33, 1694 (1939)

konzentrationen in beiden Phasen wie beschrieben ermittelten Es ergab sich für eine Anfangs-konzentration von

$$8 \cdot 10^{-3} m \quad K = 1.48 \qquad 6 \cdot 10^{-4} m: \; K = 1.79 \qquad 2 \cdot 10^{-5} m \; K = 1.33.$$

Der oben beschriebene 10-Stufen-Versuch deutet darauf hin, daß sich K auch bei Konzentra-tionen, die erheblich höher sind als diese, nicht sehr stark ändert.

Abtrennung von FAD aus einem Gemisch mit FMN

Das nach S. M. H. CHRISTIE[4] erhaltene Reaktionsprodukt aus 0.5 g Thallium-riboflavin-phosphat und der entsprechenden Menge des Adenosin-Abkömmlings wurde nach der Aufarbeitung bis zur Verseifungsstufe neutralisiert und direkt mittels *Gegenstromverteilung* fraktioniert Nach einer ersten 24-stufigen Verteilung zeigte die Kurve ein Maximum bei Rohr 13 (FMN), aber keine Inflexion im Gebiet, wo man das Auftreten von FAD hätte erwarten sollen. Jedoch enthielt eine Probe aus Rohr 7 etwas FAD, wie sich bei papierchromato-graphischer Prüfung (vgl. unten) erwies. Der Inhalt der Rohre 4—10 wurde daher vereinigt und einer weiteren, 49-stufigen Verteilung unterworfen, deren Kurve Abbildung 2 zeigt. *Die Berechnung ergab, daß etwa 1 mg FAD* (K = 0.41) *zugegen war, und daß dies 4 % des Gesamt-Flavins in dieser zweiten Verteilung entsprach.*

Die *papierchromatographische Prüfung* der aus den FAD-Spitzenfraktionen gewonnenen Substanz bestätigte, daß die Hauptmenge des vorhandenen Flavins den gleichen R_F-Wert hatte wie FAD anderer Herkunft (vergl. unten).

Untersuchung des Flavins aus Xanthinoxydase

45 ccm einer 2-proz. Lösung von Xanthinoxydase (Stufe M6[21]) in 0.5 m Phosphatpuffer vom p_H6 wurden im siedenden Wasserbad 15 Min. im Dunkeln erhitzt. Das denaturierte Pro-tein wurde abzentrifugiert und mit Pufferlösung gewaschen, darauf das Flavin aus der mit NaCl gesättigten wäßrigen Phase mit einer kleinen Menge Phenol extrahiert. Der Extrakt wurde als Teil der unteren Phase in das Rohr 0 des Apparates gegeben, worauf man 43 Überführungen vornahm Abb. 3 zeigt die Verteilungskurve. *Der Inhalt der Rohre mit den Spitzenfraktionen wurde vereinigt und einer weiteren, 43-stufigen Verteilung unterworfen; die Kurve wies keine bedeutsamen Unterschiede gegenüber der ersten auf, und der K-Wert war unverändert.*

Die Spitzenfraktion der ersten Verteilung lieferte die folgenden Absorptionsdaten: E_{265}/E_{450} = 3 34, E_{305}/E_{450} = 0 12; E_{375}/E_{450} = 0.83. Zum Vergleich seien die von L. G. WHITBY[23] für Hefe-FAD gefundenen Werte angegeben· E_{266}/E_{450} = 3 35; E_{305}/E_{450} = 0.14; E_{375}/E_{450} = 0 82 Wir haben unsere Messungen — nach vollständiger Entfernung der Phenole durch wieder-holtes Ausschütteln mit Äther — gegen eine Leerprobe der Verteilungslösungsmittel aus-geführt, die in genau gleicher Weise wie die Probe zuerst mit Methanol versetzt und dann mit Äther gewaschen worden war.

Die *papierchromatographische* Untersuchung (Dinatriumphosphat + Amylalkohol[2,24]) einer FAD-Probe, die bei einem gleichartigen Versuch isoliert worden war, ergab einen ein-zelnen Fleck vom R_F-Wert 0.41. Hefe-FAD zeigte unter ähnlichen Bedingungen R_F = 0.41 bis 0.43 und das in dem vorhergehenden Abschnitt beschriebene synthetische Produkt R_F = 0.41. Letzteres war mit etwas FMN (R_F = 0 61) verunreinigt.

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[23] L. G. WHITBY, Biochem. J. 54, 437 (1953).
[24] C. E. CARTER, J. Amer. chem. Soc. 72, 1466 (1950).

(wasserfrei) versetzt. Nach Einstellung des Gleichgewichtes hatten sich etwa gleiche Volumina beider Phasen gebildet. Der p_H-Wert der oberen Phase betrug 7.4.

Die *Gegenstromverteilungen* erfolgten in einer 50-stufigen Ganzglas-Craig-Apparatur[12]; bei einigen Versuchen wurde eine automatische Bewegungs- und Fullvorrichtung an den Apparat angeschlossen[11]. Jedes Rohr enthielt 10.0 ± 0.3ccm untere Phase. Das Volumen der oberen Phase war 10.0ccm bei Handbetrieb, 11.7ccm bei Benutzung der automatischen Füllvorrichtung. Um während der Verteilungen Verluste an unterer Phase aus der ersten Stufe auszugleichen, ließen wir nach jeder Überführung bis zu 0.2ccm dieser Phase, gleichzeitig mit der Zugabe der oberen Phase, in das erste Rohr frisch zufließen.

Vor der Einführung des zu trennenden Substanzgemisches in die Apparatur wurden mit einigen Portionen der oberen Phase, die man nacheinander in Rohr 0 einbrachte, mehrere Überführungen vorgenommen, um alle Rohre mit beiden Phasen zu benetzen; falls dies unterblieb, neigte das „führende" Rohr beim Verteilungsvorgang dazu, einen Überschuß an unterer Phase anzusammeln.

Mit Rücksicht auf die Lichtempfindlichkeit der Riboflavin-Derivate wurde während der Gegenstromverteilungen helles Licht stets vermieden und die Apparatur, wenn immer möglich, mit einem schwarzen Tuch bedeckt.

Nach einer geeigneten Zahl von Überführungen wurde die Gesamt-Substanzmenge in jedem Rohr auf Grund der Lichtabsorption bei 450mμ bestimmt, wobei jeweils 10ccm Methanol zugefügt wurden, um homogene Lösungen für die *Messungen* zu erhalten. Die theoretischen Verteilungskurven wurden mit Hilfe binomischer Wahrscheinlichkeitstabellen[22] errechnet unter Berücksichtigung kleiner Blindwerte, die von der Lichtabsorption der Lösungsmittel und den Spektrophotometerzellen herrührten. Der Blindwert wurde in jedem Verteilungsversuch aus den Ablesungen bei solchen Rohren erhalten, von denen erwartet werden durfte, daß sie keine gelöste Substanz enthielten.

In den *Abbildungen* sind die theoretischen Kurven durch ausgezogene Linien wiedergegeben; die punktierten Linien stellen die Summen sich überschneidender theoretischer Kurven dar. Die angegebenen K-Werte beziehen sich auf gleiche Volumenverhältnisse der Phasen und sind dementsprechend korrigiert[12].

Reinigung von Flavinmononucleotid (Handelspräparat)

Bei dem in Abbildung 1 dargestellten Versuch wurden 0.052g FMN (Lights, Slough/England) in 10ccm der oberen Phase des Lösungsmittelgemisches gelöst, in Rohr 0 der Apparatur eingebracht und 26 Überführungen unterworfen.

Ein weiterer Versuch wurde mit der 10fachen Anfangskonzentration an FMN derselben Herkunft durchgeführt. Nach 10 Überführungen zeigte die Verteilungskurve ein Verteilungsverhältnis von 1.94 für die Hauptkomponente an. Die Rechnung ergab, daß eine Vereinigung der Fraktionen 4 bis 7 vom FMN 69% in über 99-proz. Reinheit geliefert haben würde. Hierauf haben sich erfolgreiche Reinigungen in größerem Maßstab gründen lassen, bei denen eine Reihe von Scheidetrichtern anstelle der Verteilungsapparatur verwendet wurde.

Den Einfluß der Konzentration auf den Verteilungsquotienten einer hochgereinigten FMN-Probe haben wir bestimmt, indem wir Lösungen derselben von bekannter Konzentration in der wäßr. Phase mit gleichen Volumina der phenolischen Phase ausschüttelten und die End-

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[22] *Tables of the Binomial Probability Distribution*, National Bureau of Standards, Applied Mathematics Series 6, United States Government Printing Office, Washington 1950.

Compound	B p./mm	n_D (temp)	Formula	Required (%) C	H	Cl	S	Found (%) C	H	Cl	S	Biolog. activity
Methyl methanesulphonate †	96—98°/19 ª	1·4150 (20°)	$C_2H_6O_3S$	34·8	7·3	—	23·2	34·2	7·2	—	23·2	++ve
Ethyl	90°/10 ª	1·4194 (15°)	$C_3H_8O_3S$	39·4	8·0	—	21·1	38·9	8·1	—	21·0	—ve
n-Propyl	115°/21	1·4209 (20°)	$C_4H_{10}O_3S$	29·0	6·5	—	25·8	38·9	8·4	—	25·8	—ve
n-Butyl	118°/14 ª	1·4249 (20°)	$C_5H_{12}O_3S$	34·8	7·3	—	23·2	35·1	7·5	—	23·8	Low
Methyl ethanesulphonate *‡	100—101°/24 ª	1·4180 (18°)	$C_3H_8O_3S$	25·3	5·0	—	22·5	25·1	4·8	—	22·7	—+ve
Ethyl	108—112°/24 ª	1·4232 (18°)	$C_4H_{10}O_3S$	22·7	4·5	—	20·3	23·0	4·6	—	20·3	—+ve
2-Fluoroethyl methanesulphonate	130°/10	1·4150 (21°)	$C_3H_7FO_3S$									
2-Chloroethyl	130°/11	1·4570 (19°)	$C_3H_7ClO_3S$									
"	(b) 5—6°)	—	$C_3H_7ClO_3S$									
2-Bromoethyl	126—128°/5	1·4835 (19°)	$C_3H_7BrO_3S$	17·7	3·5	(Br, 39·3)	17·4	17·0	3·5	(Br, 38·5)	17·0	—ve
2-Iodoethyl	140°/8	1·5300 (21°)	$C_3H_7IO_3S$	14·4	2·7	(I, 32·6)	14·3	14·3	2·9	(I, 51·0)	14·5	—ve
2-Cyanoethyl	(M.p. 34—38°) ª	—	$C_4H_7NO_3S$	22·2	4·7	(N, 9·4)	21·5	32·0	4·5	(N, 9·4)	20·7	—+ve
2-Chloroethyl toluene-p-sulphonate ª	205—208°/20	—	$C_9H_{11}ClO_3S$					40·9	5·0	13·4	12·1	—ve
carboxybenzenesulphonate	(M.p. 186—188°) ª	—	$C_9H_{11}ClO_5S$	40·8	3·7	13·4	12·4	28·1	3·2			—ve
ethanesulphonate ª	128—130°/13	1·4559 (18°)	$C_4H_9ClO_3S$	27·8	5·3			32·3	4·6	19·1	16·5	—ve
propane-α-sulphonate	146°/16	1·4565 (19°)	$C_4H_9ClO_3S$	32·4	4·0	19·1	17·2	45·7	4·5	15·2	13·0	—+ve
toluene-ω-sulphonate ª	(M.p. 47°) ª	—	$C_9H_{11}ClO_3S$	46·0	4·7	15·1	13·6	45·7	3·5		14·4	—ve
Ethylene 1 : 2-di-(p-carboxybenzenesulphonate) ª	(N.) 262—264°) ª	—	$C_{16}H_{14}O_{10}S_2$	37·8	3·3		14·9	37·7	3·8		14·4	—ve
1-Chloro-2-methanesulphonyloxypropane ª	143—145°/15	1·4530 (20°)	$C_4H_9ClO_3S$	25·4	5·3	20·6	18·6	27·7	4·0	20·8	18·2	—ve
3-Chloropropyl ª	144—145°/15	1·4588 (18°)	$C_4H_9ClO_3S$	27·8	5·0	20·6	18·6	27·7	4·0	20·6	18·7	Low
3-Chloropropyl methanesulphonate	160—162°/13	1·4825 (20°)	$C_4H_9ClO_3S$	24·3	3·0	34·3	15·5	23·0	4·1	34·5	14·5	—ve
1 : 3-Dichloro-2-methanesulphonyloxypropane ª	229—230°/20 ª	1·5330 (20)	$C_4H_8Cl_2O_3S$	38·4	3·2	22·7		38·5	3·4	35·1		Low
1 : 3-Dichloro-2-toluene-p-sulphonyloxypropane ᶠ	(M.p. 206—209°) ª	—	$C_{10}H_{12}Cl_2O_3S$	19·1	3·2	(Total halogen 45·8)	12·8	19·8	3·3	(Total halogen 45·2)	13·0	+
1 : 3-Dichloro-2-p-carboxybenzenesulphonyloxy-propane	100—102°/16	1·5292 (16°)	$C_{11}H_{10}O_5Cl_2S$	16·2	2·7	(Total halogen 45·8)	10·8	16·0	2·9		11·0	Low
1-Bromo-3-chloro-2-methanesulphonyloxypropane	(M.p. 54—56°) ª	—	C_4H_7BrClS	25·9	6·2		5·2	26·0	5·2		27·0	Low
1 : 2-Dibromopropyl methanesulphonate	164—170°/15	—	$C_4H_8O_3Br_2S$	32·6	9·0			32·6	6·2			Low
Propylene 1, 2-dimethanesulphonate ª	(M.p. 106·5°) ª	1·4602 (18°)	$C_5H_{12}O_6S_2$	29·7	5·7	(N, 9·6)		29·7	2·4	(N, 8·6)		—ve
4-Chlorobutyl methanesulphonate	(M.p. 93—94°) ª	—	$C_5H_{11}ClO_3S$	35·1	6·6		26·3	35·2	6·7		26·1	—ve
2-Chloroethyl 2 : 4-dinitrobenzenesulphonate ª	(M.p. 94—95°) ª	—	$C_8H_8O_7N_2S$				23·4			(N, 8·6)	23·3	—ve
Dimethyl butane-1 : 4-disulphonate			$C_6H_{14}O_6S_2$							36·1		
Diethyl butane-1 : 4-disulphonate			$C_8H_{18}O_6S_2$									

and Voss and Blanke (*Annalen*, 1931, **485**, 258) give b.p. 101—102°/25 mm, n_D^{22} 1·4157. ² Bulleted (*Ber.*, 1905, **38**, 2018) gives b.p 85—88°/10 mm. ² Sekera ª Marvel (*J. Amer. Chem. Soc.*, 1933, **55**, 346) give b.p 105—108°/6 mm. ᵈ 1·4319 ᵉ Carus (*J. prakt. Chem.*, 1870, **2**, 270) gives b.p. 197·5—200·5°. ³ Meuwsen and Gebhardt (*Ber.*, 1937, **70**, 792) give b.p. 88·5—89°/3—9 mm ᶠ Prepared by Clemo and Perkin's method (*J. Chem. Soc.*, 1922, **121**, 642).ᵖ they give p 210°/21 mm and Gilman and Cretcher (*J. Amer. Chem. Soc.*, 1942, **64**, 1162) give n_D^{25} 1·5260. ª Prepared by the method of Sletzinger, Chamberlin, and Tishler (*ibid.*, 1952, **74**, 5619) who give b.p 78—79°/10 mm. Blanchard (*Bull. Soc. chim. France*, 1927, **41**, 834) gives b. p. 180—210°/13 mm, n_D 1·5357 ª These compounds were prepared by Dr. W. Davis ² These known compounds were prepared by our general method. ¹ Prisms from methanol ² Needles from benzene. ³ Prisms from benzene ⁴ Plates from benzene ⁵ Prisms from acetone— ether ⁶ Needles from benzene-*cyclo*hexane. ⁷ Plates from chloroform-pentane.

Some Potentially Cytotoxic Alkyl Sulphonates.

By W. C. J. Ross and (in part) W. Davis.

2-CHLOROETHYL METHANESULPHONATE is known to be effective in preventing the growth of a transplanted rat tumour [1] and to have unusual mutagenic properties.[2] Some related sulphonic esters have now been prepared for biological testing.

The 2-chloroethyl ester may be prepared by heating a mixture of ethylene chlorohydrin and methanesulphonyl chloride until no more hydrogen chloride is evolved [3] but a more general method is to treat the alcohol with the acid chloride in the presence of pyridine at low temperature. Methyl and ethyl butane-1 : 4-disulphonate, however, were more conveniently prepared from the diacid chloride and sodium alkoxide in the corresponding alcohol.

To increase their aqueous solubility, toluene-*p*-sulphonic esters were oxidised to the corresponding *p*-carboxy-derivatives which were administered as sodium salts.

Kiprijanow [4] reported that esters of 2 : 4-dinitrobenzenesulphonic acid are highly reactive as alkylating agents and this has prompted the preparation of the 2-chloroethyl ester. In this instance owing to the greater reactivity of the ester it was necessary to use 2 : 6-lutidine in place of pyridine and to carry out the reaction at −40°.

The effectiveness of the compounds as inhibitors of the transplanted Walker rat carcinoma is shown in the Table. None of the compounds is more effective than the original chloroethyl ester but methyl, ethyl, and fluoroethyl methanesulphonate are of the same order of activity when given at somewhat higher dose levels.

Experimental.—Preparation of alkyl sulphonates : general method. The alcohol (1 mole) in dry pyridine (150 ml.) and ether (150 ml.) at −10° to 0° was stirred whilst methanesulphonyl chloride (1 mole) was added during 1 hr. After being at 0° overnight the mixture was poured on ice-water containing concentrated sulphuric acid (75 ml.). An ether extract was dried (K_2CO_3–Na_2SO_4) and distilled under reduced pressure in the presence of a few pieces of potassium carbonate to prevent autocatalytic decomposition.[5] For the more reactive methanesulphonates, *e g* , those from methanol and ethanol, it was advisable to keep the temperature below −30° continuously before pouring on ice.

2-Chloroethyl p-carboxybenzenesulphonate 2-Chloroethyl toluene-*p*-sulphonate (3 g) in concentrated sulphuric acid (30 ml) and water (30 ml.) was treated slowly on a steam-bath with chromic acid (8 g.) in water (20 ml.). After ½ hr solid began to separate and after addition of water the mixture was extracted with ether (500 ml) The acid, which was extracted from the ether layer with 2N-sodium carbonate and recovered by acidification, formed prismatic needles, m p. 186—188°, from benzene The oxidation could also be carried out in glacial acetic acid without the addition of sulphuric acid 1 · 2-Di-*p*-carboxybenzenesulphonyloxyethane and 1 : 3-dichloro-2-*p*-carboxybenzenesulphonyloxypropane were similarly prepared.

2-Chloroethyl 2 : 4-dinitrobenzenesulphonate 2 4-Dinitrobenzenesulphonyl chloride (14 g.) was added during ¼ hr to a stirred solution of ethylene chlorohydrin in dry 2 . 6-lutidine (50 ml) and ether (25 ml.) at −40° After 4 hr. the temperature was allowed to rise to 0° and the mixture was poured on ice-water containing concentrated sulphuric acid (20 ml.). The *ester* which was extracted with chloroform formed yellow needles (9 6 g.), m p 104 5—106 5°, from benzene–*cyclo*hexane (1 : 3).

Esters of butane-1 4-disulphonic acid To a cooled solution of sodium (0 91 g.) in methanol (100 ml.) was added a solution of butane-1 : 4-disulphonyl chloride (5 g) in methanol (25 ml). After 1 hour's shaking at room temperature the solvent was removed under reduced pressure. The residue was extracted with chloroform (3 × 25 ml.) and on the addition of an excess of pentane the *dimethyl ester* separated as plates The *diethyl ester* was similarly prepared.

Xanthine Oxidase Activity in Normal and Abnormal Growth

BY

Dr. I. LEWIN

A. Levels of Xanthine Oxidase Activity during Normal Growth

Xanthine oxidase activity was measured in mammæ in a total of 115 C+ mice during the virgin state, pregnancy, lactation and involution. Since this organ undergoes profound changes in size, structure and function during the reproductive cycle, it was necessary to search for an expression of enzyme activity which would permit biological interpretation. Of the many indices, such as wet weight of tissue, defatted dry weight, total nitrogen, DNA and cell count, it would seem that the expression of enzyme activity as a function of the variable cellularity would best reflect the changes in a growing tissue. Also, in order to unify the presentation of the data, the observed absolute values are expressed as a percentage of the baseline value, that is, the average value obtained in breasts from up to 50 virgin mice.

1. *Changes in weight.*—In each case, the five pairs of breast pads were stripped off the underside of the skin and weighed. The tissue was then minced with fine scissors and an aliquot was removed for the determination of water, fat and defatted dry weight compositions. Another sample was taken from a pyrophosphate homogenate for total nitrogen. The results are shown in Fig. 1.

From an average value of about 400 mg. of total mammary tissue in a virgin mouse, the mammæ increase in weight to about 1,800 mg. around the middle of the lactation phase. During the first 16 days of pregnancy the increase in weight seems slower than during the last 3 days of pregnancy. It has not been estimated how much of the further weight increase beyond the moment of parturition is due to milk retained in the ducts and alveoli of the mammæ although the litter is allowed to nurse until the sacrifice of the mouse. According to Folley and Greenbaum (1947), the milk content may be between 40 and 50% of the weight of the mammæ in rats. A decrease in breast weight occurs towards the later part of lactation and is accelerated after weaning.

days of pregnancy.

[1] Haddow and Ross, *Nature*, 1956, **177**, 995
[2] Fahmy and Fahmy, *ibid*, p. 996
[3] Cf. Clemo and Perkin, *J*, 1922, **121**, 644
[4] Kiprijanow, XIVth Internat Cong Pure Appl. Chem. Handbook, 1955, p. 320.
[5] Cf. Scott and Lutz, *J Org Chem*, 1954, **19**, 830

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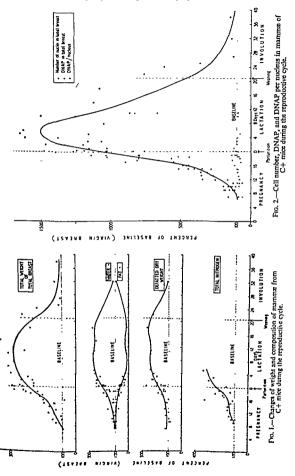
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The wet weight can be thought of as being the sum of the amount of water, fat and defatted

The amount of total nitrogen per mg. of fresh tissue also increases during pregnancy and continues to do so after parturition, which may be a reflection of milk production and retention. In regard to water, fat and nitrogen content one can distinguish a slow phase of change (up to about the 16th day of pregnancy) and an accelerated phase during the last 3 days of pregnancy.

(470)

Fig. 2.—Cell number, DNAP, and DNAP per nucleus in mamma of C+ mice during the reproductive cycle.

Fig. 1.—Changes of weight and composition of mamma from C+ mice during the reproductive cycle.

II. *Changes in cellularity.*—The change in weight of the total ____

seen to be the resul
change in tissue me
per unit of weight
fat and water cont___ __
later part of pregnancy ar
tration, the total number ___
in a sample from a sucrose-CaCl₃ homogenate were counted in a hæmacytometer and the number of nuclei per mg. of wet tissue were multiplied by the total weight of the mammæ in a mouse.

In the majority of samples, the sucrose-CaCl₃ homogenate was also used for DNA

reagent. The colour was read at 600 mμ in a *SP 500 Unicam Spectrophotometer* and compared with a DNA standard.

Fig. 2 shows the great increase in total cell numbers from about 30×10^9 in a virgin breast to a maximum of 450×10^9 at around the 4th to 6th day of lactation. The increase in cells is greater (about fifteen-fold) than the increase in weight (about four-fold). This is real

not used by the litter.

The amount of DNAP (desoxyribose nucleic acid phosphorus) in the total breast as well
the c
duri
on r
(195
DNA
of th

The series here reported supports the observations that the mammæ continue to grow

However, Turner has stated that in mice the hyperplasia of the mammary gland parenchyma is complete by the 12th day of pregnancy (Turner and Gomez, 1933) and that in rats the amount of DNA content in the inguinal breast pads has reached a maximum at about the 10th day of pregnancy after which it remains level until the time of weaning (Kirkham and Turner, 1953). No explanation can be offered for these differences, except that they may be due to differences in strain and species.

III. *Changes in xanthine oxidase activity of mammary tissue.*—The xanthine oxidase activity of the homogenate was determined by measuring the rate of uric acid formation. The rate per mg. of wet tissue was related to nuclear count. defatted dry matter and total nitrogen per mg. of wet tissue; thus eliminating the variations introduced by the changes in water and fat content of the mammæ during pregnancy, lactation and involution.

shortly before parturition; this activity drops precipitously to about half this value for the
duration of lactation. A decrease in xanthine oxidase activity becomes noticeable a few
eaning.
trogen)

The peak in enzyme activity just before parturition is surprising and needs further comment.

IV. *Xanthine oxidase activity in milk.*—One of the earliest and richest sources of this enzyme is cow's milk. It was therefore essential in this investigation to evaluate the effect

of mouse milk on the determination of xanthine oxidase in mammæ of mice during late pregnancy and lactation. It was noted that in some mice the mammæ were completely filled with milk, appearing engorged and white, whilst in other animals of the same day in pregnancy or lactation the mammæ were entirely pink and presumably containing not so

Fig. 3.—Xanthine oxidase activity in mammæ during the reproductive cycle.

much milk. However, there was no consistent or significant difference in the xanthine

TABLE I.—COMPARISON OF XANTHINE OXIDASE ACTIVITY IN MAMMÆ CONTAINING DIFFERENT AMOUNTS OF MILK

Tissue	Appearance	Weight (mg.)	Amount of milk removed (ml.)	Nuclei/mg. of wet tissue ($\times 10^6$)	Micromoles ($\times 10^{-4}$) of xanthine oxidized/minute per mg. wet weight	10^6 nuclei
Breast pads of the right side (control)	White; engorged with milk	1,210	0·0	151	32·3	21·4
Breast pads of the left side ("milked")	Entirely pink	800	0·341	184	39·2	21·3

per nucleus while the activity per mg. of wet tissue is the "milked" mammæ. Had the milk contributed to uld have expected the homogenate of the right breast ity per nucleus and to have the same activity per mg. when compared to the left mammæ.

At this point it should be pointed out that the tissue homogenate is centrifuged at 20,000 × g for 30 minut⋯⋯⋯ ⋯ ⋯ ⋯ ⋯ ⋯ 37° C. incubator prior ⋯

anæsthesia 1 ml. of Pitocin containing one international oxytocic unit was injected

⋯⋯ ⋯⋯⋯⋯⋯ ⋯⋯⋯⋯ ⋯⋯⋯⋯ ⋯⋯ ⋯⋯⋯ ⋯⋯⋯⋯⋯ (Table II). This is in contrast to the

TABLE II.—Effect of Incubation and Centrifugation upon the Xanthine Oxidase Activity of Breast Tissue and Mouse Milk

		Micromoles (× 10⁻⁵) of xanthine oxidized/min /mg. wet weight	
Material	Centrifugation	No incubation	Incubation
Mammary tissue	600 × g; 10 min.	21·3	49·8
	20,000 × g; 30 min.	—	47·7
Milk	None	17·5	18 6
	20,000 × g; 30 min.	—	0 0

tissue homogenate where enzyme activity is increased by incubation but not materially altered by centrifugation.

This difference in response to the centrifugation nearly eliminated the so called "milk

TABLE III.—Xanthine Oxidase Activity in Mixtures of Mammary Tissue and Mouse Milk after Centrifugation and Incubation

Preparation No.	Mammary tissue (g.)	Mouse Milk (g.)	Tissue concentration (%)	Micromoles (× 10⁻⁵) of xanthine oxidized/ min /mg wet weight	Enzyme activity as percentage of Preparation No. 1
1	0·3781	0 0	100·0	47 7	100·0
2	0 1441	0·0658	68·8	34·2	71 8
3	0·1009	0·1044	53·5	24 6	51·5
4	0·0550	0 1494	26 9	9·1	19·1
5	0·0	0 3712	0·0	0 0	0 0

in terms of the tissue enzyme alone.

of mouse milk on the determination of xanthine oxidase in mammæ of mice during late pregnancy and lactation. It was noted that in some mice the mammæ were completely filled with milk, appearing engorged and white, whilst in other animals of the same day in pregnancy or lactation the mammæ were entirely pink and presumably containing not so

FIG. 3.—Xanthine oxidase activity in mammæ during the reproductive cycle.

much milk. However, there was no consistent or significant difference in the xanthine oxidase activity either per unit of weight or per cell between the homogenates from presumably "milk-rich" and "milk-poor" mammæ. In order to follow up this observation a mouse on the 8th day of lactation was anæsthetised from its litter for four hours. During this period milk was allowed to accumulate in the mammæ on the right side of the animal, whilst those on the left side were hand-milked repeatedly so as to keep them as empty as possible. At the end of this period the mammary tissue was excised and examined.

TABLE I —COMPARISON OF XANTHINE OXIDASE ACTIVITY IN MAMMÆ CONTAINING DIFFERENT AMOUNTS OF MILK

Tissue	Appearance	Weight (mg.)	Amount of milk removed (ml.)	Nuclei/mg. of wet tissue (× 10⁶)	Micromoles (× 10⁻⁴) of xanthine oxidised/minute per mg.	
					wet weight	10⁶ nuclei
Breast pads of the right side (control)	White, engorged with milk	1,210	0·0	151	32·3	21·4
Breast pads of the left side ("milked")	Entirely pink	900	0·341	184	39·2	21·3

Fig. 4 —Xanthine ... low tumour strain (C
(C+) with milk facto.
(C+) mice; 24 anima... ... ...

II. *Xanthine oxidase activity in liver tumours.*—These experiments were performed in
... ... mours in rats by means
... ... data are presented in
... ... the enzyme activity is
expressed per mg. of wet tissue. Because of the small number of observations a statistical
analysis of the difference of the means was not performed, but the graph reveals no overlap
of the individual values comprising each group.

There is good agreement between the observations here reported and those previously
published involving different techniques of assay, different strains and different tissues.

Weiler (1956) was able to show by an ingenious histo-serological technique the loss of a liver-
specific antigen during azo-dye carcinogenesis. However, β-glucuronidase has a higher

Growth in the case of the tumour means no more than cell multiplication resulting in an
increased tissue mass, whereas in the mammæ there is also a change in function besides the
increase in cellularity. Since growth can proceed without an increase in xanthine oxidase

was the third of the breast used periods... The factors ... conditions, however, was similar to the methods used at the height of its action ... significance and ... with the appearance of the xanthine oxidase activity of the mammary tissue. The possible significance, therefore, for the peak in xanthine oxidase activity period suggests more detailed research on known forms of enzyme ... of the enzyme production of the mammary gland during ... enzyme. The disappearance of this peak during lactation would thus reflect the disappearance of this ... in ... enzyme production and secretion.

A further study was undertaken ... Pregnancy and lactation are associated with pronounced anatomical and metabolic changes. It was found... concentration during ... the mammary tissue in regard to xanthine oxidase activity was low in the milk cows by weaning ... a rise in the enzyme concentration ... Maternal JK and ... 1890 ... The average level of xanthine oxidase activity was compared in the different states through the reproductive cycle and which comprised the five forms from virgin mice ... It was seen that while the enzyme activity per gram in virgin (C+) primiparous and multiparous ... the virgin state, the enzyme again reached maximum activity during the breast tissue from the liver during late pregnancy and lactation ... There is a small but significant age-bound increase in the total number of liver cells during pregnancy, lactation and the observed peaks disappear at weaning.

B. Testis or Xanthine Oxidase Activity of the Abnormal Glands

The increase in the number of mammary cells during the late menstruating period is associated with an increase in xanthine oxidase activity per cell. Two questions arise from this observation. (1) What are the levels of xanthine oxidase activity in tumours as compared to the tissue of origin? (2) What is responsible for the observed changes in the growth of tumours?

The data reported in the following section have been collected in an attempt to answer these questions.

1. Xanthine oxidase activity in spontaneous breast tumours. The design of the experiment entailed a comparison of xanthine oxidase activity in mammary tissue and in mammary tissue from cancerous strains of mice and cancer-resistant mice of the same strain. Inbred mice of the C+ strain constituted the cancer-prone group... Three mice of this group have the high age-still tumour factors and develop mammary tumours spontaneously at a high rate during pregnancy. Cancer concept which was employed in this C+ strain for the enzyme study of JK mice, then incorporating the milk agent... Mice of this strain develop a low, long-called C+ rarely develop mammary tumours if they are virgin. In this group of tumours a low rate at an average age of 2 months of longevity. The material for the tumour series was obtained from these C+ mice.

The results are summarized in Fig. 4. The differences in the average levels of enzyme activity per cell are significant, $p < 0.001$ when comparing group a and c to b. Relative to the enzyme activity of breasts from C+ mice, the activity in breasts from C+ mice is 57.7%, and in tumours 24.5%. It should be stressed that the breasts in group a derive from animals that had no tumour and were 25 at a full rest developing tissue. The difference in xanthine oxidase activity in the tumours... and JK... we can therefore be related to the absence or presence of the milk agent or the complement, and not to the genetic constitution of the actual presence of a tumour. The enzyme activity in the normal parts of the breasts from mated C+ mice with tumours is similar to that in breasts of C+ virgin mice.

In contrast to the enzyme in the breast, the xanthine oxidase activity in livers of C+ and C+ mice does not differ materially. This is in line with the observation that the liver contains relatively small amounts of each agent. Moreover, Figge and Strong (1941) found a marked difference in xanthine oxidase activity of livers from mice with a high and mice with a low incidence of breast tumour, the CJH and JK strains respectively. But the design of their experiment makes it difficult to decide whether the difference in enzyme activity reflects a difference in cancer-susceptibility or in genetic constitution.

The tumours used ranged from 0.3 grams to 7.0 grams and could be divided into solid, firm grey and soft, haemorrhagic purplish tumours. Histologically, they were all adenocarcinomas but showed varying degrees of dedifferentiation. For purposes of the assay necrotic material was avoided. There was no significant difference in the enzyme activity of the different kinds of tumours. Each average mammary cancer cell possesses one-fourth the xanthine oxidase activity contained in the average normal mammary cell and one-half the activity present in a precancerous breast cell (a cell infected with the milk agent).

Since there is no statistically significant difference in the cellularity of breasts from virgin mice of the C+ and C- strain, it is concluded that the xanthine oxidase activity is lowered in the precancerous state, whether expressed as enzyme concentration (activity per mg.) or as content (activity per cell).

Xanthine Oxidase Activity during Mammary Carcinogenesis in Mice

SOME hypothetical and empirical considerations that form the basis of our interest in xanthine oxidase have been stated in the first of a series of publications[1]. The investigations of the chemical properties of xanthine oxidase[1-3] have been extended to include the biological behaviour of this enzyme in normal and abnormal growth. The present communication deals with the xanthine oxidase activity in mammæ of mice of the C strain, with and without the milk factor, and in spontaneous mammary tumours. The studies of the enzyme activity in mammæ during pregnancy and lactation will be reported elsewhere.

The mice of the C strain without the milk factor will be referred to as C −. A sub-line of these mice (C +) was developed by incorporation of the milk factor through foster-nursing. The tumour incidence in C − virgins and breeders is practically nil, whereas the tumour incidence in C + mice, while low in virgins, rises progressively to 100 per cent with the number of pregnancies. The spontaneous mammary tumours assayed were obtained from the C + breeders.

The tissues were dispersed in a Potter–Elvehjem type of homogenizer with a Teflon pestle. The suspension medium (in the proportion of 9 ml. for each gm. of tissue) for the enzyme assay was 0·1 M pyrophosphate buffer (pH 8) and for the nuclear

and the clear supernatant was removed, avoiding the upper layer of fat, and incubated for 1 hr. at 37° C.

The enzyme assay[1], a modification of Kalckar's[1] method for determining purines, consisted in the spectrophotometric estimation of the rate of appearance of uric acid in the presence of xanthine ($2\cdot6 \times 10^{-4}$ M) as the substrate. Readings were obtained for 10 min. at 1-min. intervals at a constant temperature of 24° C. The enzyme activity was related to the cellularity of the tissue by dividing the activity per mgm. by the number of nuclei in a mgm. of fresh tissue. The nuclei were counted in a hæmocytometer after suitable dilution in a solution of orcein-calcium chloride homogenate in a solution of orcein-light green stain.

The results are summarized in Fig 1, which shows the group averages and standard deviations of the enzyme activities in mammæ from C − virgin mice (group A), from C + virgin mice (group B), and mammary tumours from C + breeders (group C). The amount of xanthine oxidized (or uric acid appearing) in micromoles × 10⁻⁴ per min. per 100,000 nuclei is 7·2, 4·1 and 1·8 for groups A, B and C, respectively. Relative to the xanthine oxidase activity in breasts from mice without the milk factor (group A), the activity in mammary tissue from mice with the milk factor (group B) is 57·7 per cent and in spontaneous mammary tumours (group C) 24·9 per cent. The differences of the average levels of enzyme activity

are statistically highly significant, the P value being less than 0·001 by the t-test.

It is of interest to mention that the xanthine oxidase activity in mammæ from multiparous, cancer-prone C + breeders, whether they have or have not yet developed mammary carcinoma, is practically the same as in mammæ from C + virgins which usually do not develop tumours. No appreciable difference in xanthine oxidase activity could be detected in livers from C − and C + mice. In contrast to the progressive decrease in the activity of xanthine oxidase associated with the events which predispose to and finally result in abnormal growth (Fig. 1), it was found that during pregnancy the enzyme activity in the growing breast pads of C + mice increased markedly.

The interpretation of the results shown in Fig. 1 is as yet difficult, since a number of conditions must be considered which need clarification. The activity measured by enzyme assays *in vitro* does not necessarily reflect the biological activity within the intact cell, since such limiting factors as substrate
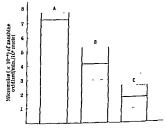
Fig. 1   Xanthine oxidase activity during carcinogenesis in mouse breast. *A*, Virgin breast from non-tumour strain (C −) without milk factor; 25 animals   *B*, Virgin breast from high-tumour strain (C +) with milk factor; 50 animals free of tumours. *C*, Spontaneous mammary tumour from mated (C +) mice; 24 animals. (Dotted line indicates ± S D)

concentration, the nature and concentration of activators and inhibitors *in vivo* are not known. Moreover, the activity per cell, as here expressed, is an average value for all cells in the homogenate and does not reveal localized changes in enzyme activity. A spotty distribution of foci of cells with low or no enzyme activity surrounded by normal cells would be possible and has been reported for antigenic cell constituents[4]. Furthermore, it should be pointed out that, although the C − and C + mice originated from . . . . -–.--ad inbreeding within . . . . ifference.
. . . . .tivity in
mammæ of group A and B [Fig. 1] . . . . it be due solely to the incorporation of the milk factor.
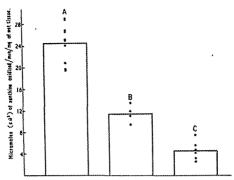
FIG. 5.—Xanthine oxidase activity during carcinogenesis in rat liver. A, Normal liver from rats on normal diet. B, Tumour-free liver from rats fed azo-dye for a short period. C, Liver tumour in rats fed azo-dye. (Dots indicate individual values.)

activity per cell, as in the mammary tumour, or even in the virtual absence of this enzyme, ~~..............~~ mammæ is best related to ~~.............~~ a product so rich in this ~~.............~~ cell of origin is the same for the lactating mammæ and the mammary cancer, the failure of the tumour to increase its level of enzyme activity is just another expression of the loss of specialized function of this dedifferentiated tissue.

REFERENCES

C~~....~~, H. A. ~~(1933)~~ ~~....~~ ~~...~~ ~~...~~ B. 114, 136

~~..............~~, ~~..~~, WELLS, ~~..~~ C., RICHERT, D. A., and WESTERFELD, W. W. ~~(1951)~~ *J. biol. Chem.*, 192, 651.

~~......~~ D. D. ~~(1956)~~ ~~...~~

press
WILSON, M. E (1954) *Arch. Biochem.*, 48,

~~......~~ C. W. ~~...~~ ~~...~~ ~~...~~ ~~(1933)~~ ~~...~~

MODE OF ACTION OF ALKYLATING AGENTS: FORMATION OF S-ETHYLCYSTEINE FROM ETHYL METHANESULPHONATE IN VIVO

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T⸻ ester ⸻ have ⸻ compounds such as amino-acids, proteins and nucleic acids, and to inhibit certain enzymes[2]; however, while work has been done on the fate of some of these compounds[3-5], little appears to be known about the precise nature of the metabolic products formed in vivo. In this connexion we wish to report the preliminary results of studies which are being carried out at this Institute to determine the mode of action of cytotoxic agents. In the first instance we studied the metabolic fate of ethyl methanesulphonate (CB. 1528) (I), one of a series of cytotoxic alkylating agents studied by Haddow and Ross[8]. It has been shown to have some inhibiting effect on the growth of the transplanted Walker rat carcinoma 256[8] and to cause mutations in *Drosophila melanogaster* (Fahmy, O. G., personal communication). It was regarded, therefore, as having properties sufficiently typical of the biological alkylating agents to warrant the present study.

$$CH_3.SO_2.O.C_2H_5$$
(I)

Although the bifunctional alkylating agents normally manifest some of their typical biological effects at approximately 1/50 the concentration of the monofunctional compounds[?], it was felt that the number and complexity of reactions which they could undergo in vivo would prevent a facile determination of the nature of the metabolic products formed. It is reasonable to suppose, however, that the monofunctional alkylating agent chosen for these studies would undergo the same initial metabolic reaction as the difunctional ones.

⸻ the ⸻ mop α ⸻ was w ⸻ ulthy in ⸻ 'istar a.

rats, and three consecutive twenty-four hour samples of urine were examined using the techniques of paper chromatography with subsequent autoradiography of the paper chromatograms, and ion-exchange chromatography. 12 per cent of the total radioactivity was excreted within the first 24 hr., and 4 per cent during the next 48 hr. Negligible quantities were excreted from then on. This radioactivity is associated with new compounds, since any unreacted drug or volatile material present in the urine would not be detected by the counting methods so far employed.

Traces of the radioactive material excreted in the first twenty-four hours were shown to be incorporated into the amino-acids serine, alanine, and arginine, and into urea. It seems likely that radiocarbon was incorporated into these compounds following hydroly-

sis of the drug into ethanol, which was then involved in biosynthetic reactions. This view was confirmed ⸻

liberated by hydrolysis of all the injected drug. Radioactivity was found only in amino-acids and urea.

The bulk of the radioactivity was shown by autoradiography of one dimensional paper chromatograms to be present in two sulphur-containing acids, one of which is S-ethyl-N-acetylcysteine (II; $R=COCH_3$) and the other possibly the keto acid (III)[8]. Autoradiography of two-dimensional chromatograms of urine hydrolysed with acid indicated that almost all the radioactivity was embodied in S-ethylcysteine (II; $R=H$). The identity of this compound was established by its R_F value in four solvent mixtures, its decomposition with hot alkali, and its behaviour on ion exchange resin and deactivated charcoal columns, in which respects it was identical with an authentic sample of S-ethylcysteine.

$$C_2H_5.S.CH_2.CH.COOH \qquad C_2H_5.S.CH_2.CO.COOH$$
$$\qquad NHR$$
(II) (III)

Two other faint areas of radioactivity which gave brown colours with ninhydrin in the presence of dicyclohexylamine were also present on this chromatogram. These areas appear to be identical with two such new brown spots which appeared on a similarly obtained chromatogram of urine from rats injected with unlabelled S-ethylcysteine, and may therefore be presumed to be derived from it, possibly by a transethylation reaction.

No evidence has been found for the presence in the urine of labelled carboxylic or phosphoric esters, or alkylated amines (amino-acids, purines, etc.), which would be formed if the drug reacted with acids or amines in vivo. However, the possibility of their being formed is not precluded, since they could be further metabolized prior to excretion.

The formation in vivo of S-ethyl-N-acetylcysteine (ethyl-mercapturic acid) from ethyl methanesulphonate is the first demonstration of the formation of mercapturic acid from an aliphatic compound. Previously mercapturic acids have been shown to be formed from a variety of aromatic compounds such as the halogenobenzenes and the hydrocarbons anthracene and naphthalene[9]. The importance of the formation of mercapturic acid from a cytotoxic compound is difficult to assess until some of the more obvious experiments have been carried out. Ethionine (S-ethyl-homocysteine) has been shown to inhibit tumour growth[10], possibly because of its ability to inhibit transmethylation reactions[11] and to interfere with protein synthesis[12]. In consequence, the tumour growth-inhibiting properties of S-ethyl-cysteine and other S-substituted cysteines which could be formed from other cytotoxic compounds is being studied by Prof. A. Haddow at this Institute.

Work is being continued to determine whether S-ethylcysteine is formed only from exogenous cysteine, or whether reaction with tissue sulphydryl groups is implicated[13], and possibly responsible for the observed biological effects. Reaction with tissue sulphydryl groups is likely to occur on the basis of the reaction demonstrated between other alkylating

While the above considerations must be borne in mind and will lead to further experiments, the present results indicate a progressive decrease of xanthine oxidase activity during carcinogenesis in the mammæ of mice carrying the milk factor. This observation is similar to the one by Westerfeld et al.[6], who noted a decrease of xanthine oxidase in livers—as yet free of tumours—from rats fed a carcinogenic azo-dye. The gradual depletion of various other intracellular constituents in liver during azo-dye carcinogenesis has been reported and the degree of loss has been correlated with the degree of carcinogenicity of the compound[7-9]. Thus, the gradual decrease of xanthine oxidase activity during the changes of the normal tissue to a precancerous and eventually to a tumorous condition may be a further instance tending to support the deletion hypothesis of carcinogenesis[10,11].

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[1] Avis, P. G., Bergel, F., and Bray, R. C., J. Chem. Soc., 1100 (1955)
[2] Avis, P. G., Bergel, F., Bray, R. C., James, D. W. F., and Shooter, K. V., J. Chem. Soc., 1212 (1955)
[3] Avis, P. G., Bergel, F., and Bray, R. C., J. Chem. Soc., 1219 (1956).
[4] Kalckar, H. M., J. Biol. Chem., 167, 429 (1947).
[5] Weller, E., Z. Naturforsch., 11b, 31 (1956).
[6] Westerfeld, W. W., Richert, D. A., and Hilfinger, M. F., Canc. Res., 10, 486 (1950)
[7] Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., Canc. Res., 9, 398 (1949)
[8] Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., Canc. Res., 10, 18 (1950).
[9] Potter, V. R., Price, J. M., Miller, E. C., and Miller, J. A., Canc. Res., 10, 28 (1950).
[10] Miller, J. A., and Miller, E. C., "Adv. Canc. Res.", 1, 339 (1953).
[11] Haddow, A., Ann. Rev. Biochem., 24, 689 (1955).

STRUCTURE-ACTIVITY RELATIONS IN TWO NEW SERIES OF ANTIFOLIC ACIDS

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ANTIFOLIC acids† are clinically useful in acute leukaemia (aminopterin, A-methopterin) and in malaria (proguanil, pyrimethamine); but aminopterin and its relatives possess the disadvantages of producing severe side-effects and of evoking drug-resistance[1], which latter can also be a problem with the antifolic antimalarials[2]. It therefore seemed useful to search for new types of antifolic acids, which might allow the development of additional therapeutically useful substances, not cross-resistant with existing types. Taking the 2:4-diaminopteridines known to possess very high antifolic acid activity in various bacterial systems[3-6] as a starting point, two series of compounds—the aryl-azopyrimidines and 8-azapurines —were prepared and investigated. Since both series were readily varied chemically, they allowed structure-activity relations to be explored in some detail. In the account that follows the chemical and biological investigations are described in separate sections and the results then brought together in a discussion of structure-activity relations.

This work was started in the Autumn of 1953, and some of the compounds were described[7] at the International Chemical Congress in 1955, where conversation with Dr. E. J. Modest revealed that somewhat later than ourselves he had prepared and begun to examine some similar compounds. Arrangements were made with Dr. Modest for simultaneous publication and a paper by himself and his colleagues[8] appears alongside our own.

CHEMICAL SECTION‡

INTRODUCTION

Antagonists of folic acid in bacterial systems have been found by various workers amongst the following simple derivatives of 2:4-diaminopteridine; 6:7-diaryl- (I, $R' = R'' = $ aryl)[3,4], 7-amino-6-aryl- (I, $R' = $ aryl, $R'' = NH_2$)[9] and 6:7-dialkyl- (I, $R' = R'' = $ alkyl)[5,6]. As a further simplification of structure we first made 2:4-diamino-5-4'-chlorophenyl-azo-6-dimethylaminopyrimidine (II, R ⩵ Cl) (No. 24) since this would exist in the *trans* form[10] (as shown) and would therefore show some spacial similarity to a 2·4-diamino-6-chlorophenylpteridine. The chlorine

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† The term antifolic acid is used to refer to an antagonist of any vitamin of the folic acid group

‡ By G.M.T. and D.G I F.

agents, such as the sulphur mustards, and protein sulphydryl groups under physiological conditions[14].

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ch
dimesyloxybutane ('Myleran') and p-N,N-di-2'-chloroethylaminophenylbutyric acid (CB. 1348), are in progress. Preliminary results have shown that in some cases additional sulphur-containing compounds are excreted in the urine.

Further consideration of the above finding could

inhibits the growth of the transplanted Walker rat carcinoma 256[5] and is atypical of mesyloxy compounds in its mutagenic effects on Drosophila melanogaster[15]. Reaction with cysteine in vivo in an analogous manner with that found in the case of CB. 1528 should in the first instance give S-chloroethylcysteine (V) (or a derivative), a monofunctional sulphur mustard. Full details of this work will be published elsewhere.

Cl CH₂ CH₂.O.SO₂.CH₃   Cl.CH₂.CH₂.S.CH₂ CH.COOH

$$\text{Cl CH}_2\text{ CH}_2.\text{O.SO}_2.\text{CH}_3 \qquad \text{Cl.CH}_2.\text{CH}_2.\text{S.CH}_2\ \underset{\overset{|}{\text{NH}_2}}{\text{CH.COOH}}$$

(IV)   (V)

[1] Haddow, A., and Timmis, G. M., Lancet, i, 207 (1953).

[2] For the references see the reviews by Phillips, F. S , Pharmacol Res , 2, 281 (1950), and Ross, W. C. J., "Advances in Cancer Research", 1, 397 (Acad. Press, New York, 1953)

[3] Boursnell, J. C., Cohen, J. A., Dixon, M., Francis, G. E , Greville, G. D., Needham, D. M., and Wormall, A., Biochem. J., 40, 756 (1946).

[4] Craig, A. W., and Jackson, H., Biochem. J., 58, xxxiii (1954).

[5] Goldenthal, E. I., Nadkarni, M. V., and Smith, P. K., Proc. Amer Assoc. for Cancer Research, 2, 110 (1956).

[6] Haddow, A., and Ross, W. C. J., Nature, 177, 995 (1956).

[7] Biesele, J. J., Philips, F. S., Thiersch, J. B., Burchenal, J. H., Buckley, S. M., and Stock, C. C., Nature, 166, 1112 (1950). Loveless, A., and Ross, W. C. J., ibid., 166, 1113 (1950).

[8] Blanchard, M., Green, D. E , Nocito, V., and Ratner, S., J. Biol. Chem., 155, 421 (1944).

[9] Williams, R. T., "Detoxication Mechanisms", 60 (Chapman and Hall, Ltd., 1947).

[10] Levy, H. M., Montanez, G., Murphy, E. A., and Dunn, M. S., Cancer Res., 13, 507 (1953). White, L. P., and Shinkin, M. B., Cancer, 7, 867 (1954).

[11] Simmonds, S., Keller, E. B, Chandler, J. P., and du Vigneaud, V., J. Biol. Chem., 183, 191 (1950)

[12] Simpson, M. V., Farber, E., and Tarver, H., J. Biol. Chem., 182, 81 (1950) Levine, M., and Tarver, H., J. Biol. Chem , 192, 885 (1951).

[13] Conway, W. J., J. Biol. Chem , 121, 27 (1937) Gutmann, H. R., and Wood, J. L., Cancer Res , 10, 8 (1950).

[14] Banks, T. E., Boursnell, J. C., Francis, G. E , Hopwood, F. I., and Wormall, A., 40, 745 (1946) Bacq, Z. M., Experientia, 2, 349 (1946). Peters, E. A., and Wakelin, R. W., Biochem. J., 41, 550 (1947).

[15] Fahmy, O. G., and Fahmy, M. J., Nature, 177, 996 (1956).

·In order to observe the effect of changing the position of the aryl substituent in the 8-azapurine series some 9-aryl derivatives were made.

9-Phenyl-2-amino-8-azapurine (VII, $R' =$ H, $R'' =$ Ph) was synthesised· from 2-amino-4-chloro-5-nitropyrimidine by condensation with aniline to form 2-amino-4-anilino-5-nitropyrimidine, reduction, and ring-closure of the 4:5-diamine by treatment with nitrous acid. Similarly were prepared the p-chlorophenyl analogue (VII, $R' =$ H, $R'' =$ $C_6H_4Cl(p)$) and the 6-methyl derivative (VII, $R' = CH_3$, $R'' =$ Ph).

(I)   (II)   (III)

(IV)   (V)   (VI)

(VII)   (VIII)

(IX)   (X)   (XI)

From structural analogy, the two pyridine-containing structures VIII and IX were potentially interesting especially since the benzeneazo analogue of IX (2-benzeneazo-2:6-diaminopyridine, "Pyridium") is a powerful bactericide. These compounds (Nos. 66 and 60 respectively), which were synthesised by methods exactly analogous to those used for the azopyrimidines and 8-aryl-8-azapurines, were inactive.

6-Thio-8-aryl-8-azapurines were made by thiation of the 6-hydroxy analogues with phosphorus pentasulphide in boiling pyridine solution. 2:6-Diamino-8-azapurine (VI, $R' = R'' = NH_2$) (No. 65) made according

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substituent in the benzene ring was selected in the hope that it would have a favourable effect because of the marked enhancement of antifolic activity in the 4:6-diamino-1-aryl-1:2-dihydro-5-triazines by the introduction of halogen substituents in position 3' or 4' or 3' and 4' of the benzene ring[11]. The discovery of antifolic activity in the azo-pyrimidine (II, R = Cl) encouraged further exploration using a diethanolamino- and morpholino-, instead of the dimethylamino- substituent but when it was found that much greater activity arose from using an unsubstituted amino group the series III was explored in detail. In the pyrimidine ring, variations involved the hydroxy, amino, thio, chloro and 4'-chlorophenylthio substituents and in the benzene ring chloro, bromo, nitro, methoxy and ethoxy substituents were used. In place of the benzene ring naphthalene and quinoline were introduced. Since amino-aryl-azopyrimidines (III) are, in general, easily oxidised to 8-azapurines[12] (IV) it was thought that this conversion might occur *in vivo* and that the 8-azapurines might therefore be active. Many active compounds were found in this series but the evidence, given later in this paper in the discussion of structure-activity relations, is against the hypothesis that the aryl-azopyrimidines owe their activity to this conversion. The 8-aryl-8-azapurines were all made by a general method[12] from the aryl-azopyrimidines (III) which were usually synthesised by the method[12] of coupling the appropriate pyrimidine and aryl diazonium salt. The azo compounds were oxidised by copper sulphate in boiling pyridine, the yields being greatly improved by passing oxygen through the reaction mixture. Azo compounds containing a tertiary amino group in the 4- (or 6-) position of the pyrimidine, e.g., II, were made by reacting the appropriate chloro-aryl-azopyrimidine (X) and secondary amine.

The dimethylamino derivatives (II, R = Cl and NO₂) were also formed by reacting the chloro-pyrimidines (X, R' = Cl and NO₂, R" = Cl) and guanidine in a mixture of ethanol and dimethylformamide, respectively under hot and cold conditions. Presumably the reaction of dimethylformamide and guanidine liberates dimethylamine. From X, (R' = NO₂, R" = Cl) in this reaction there is also formed 2·4-diamino-6-ethoxy-5(4'-nitrophenylazo)pyrimidine (X, R' = NO₂, R" = OEt) but none of the corresponding ethoxy derivative could be isolated from the chlorophenyl analogue (X, R' = Cl, R" = Cl). From X, (R' = Cl, R" = Cl) and hot 2-ethoxyethanol in the presence of guanidine the

type of reaction. When 4.6-diamino-2-thiopyrimidine was coupled with *p*-chlorobenzene diazonium chloride the desired 4:6-diamino-5(4'-chlorophenylazo)-2-thiopyrimidine (XI, R = SH) was formed in small yield, the major product (XI, R = SC₆H₄Cl (*p*)) having been formed by coupling with two mols of the diazonium chloride. Previously[13] 2-thiouracil and 4-methyl-2-thiouracil have been coupled with this diazonium chloride but yielded only the product derived from two mols of the reagent; in our case the 4.6-diamino groups therefore appear to direct the coupling to the 5-position more strongly.

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of dimethylamine (33 per cent, 3 ml.) and the mixture was heated under reflux at 100° when the azo compound passed into solution. After one hour, water was added to turbidity and the solution filtered. *On cooling*, the solution deposited *dark maroon crystals* with a green lustre (0·87 g.), which after recrystallisation from aqueous 2-ethoxyethanol had m.p. 252 to 255°, undepressed on admixture with a sample prepared according to Method 2.

Method 2. 2:4-Diamino-6-chloro-5(4'-nitrophenylazo)pyrimidine (1 g.) was dissolved in warm dimethylformamide (20 ml.) and treated with a solution of guanidine (prepared from guanidine hydrochloride (0·95 g.) and sodium (0·23 g.) dissolved in ethanol (20 ml.), with removal of the precipitated sodium chloride). There was an immediate precipitation of crimson solid, which was allowed to stand overnight. The precipitate (1 g.) was then collected and fractionally crystallised from aqueous 2-ethoxyethanol to yield :—

(i) *2:4-Diamino-6-dimethylamino-5(4'-nitrophenylazo)pyrimidine.* This formed the less soluble fraction and recrystallised from aqueous 2-ethoxyethanol as deep maroon leaflets with a strong metallic green lustre, m.p. 252 to 255°. (Found: C, 48·0; H, 4·6; N, 37·45; $C_{12}H_{14}O_2N_8$ requires C, 47·7; H, 4·6; N, 37·1 per cent). This was sparingly soluble in dilute hydrochloric acid and on examination in ultra-violet light, the solution had a light blue fluorescence.

(ii) *2:4-Diamino-6-ethoxy-5(4'-nitrophenylazo)pyrimidine.* This was precipitated on dilution of the mother liquors from which (i) had separated. It recrystallised from aqueous 2-ethoxyethanol as deep orange needles, m.p. 214 to 215°. (Found: C, 47·7; H, 4·6; N, 32·0. $C_{12}H_{13}O_3N_7$ requires C, 47·5; H, 4·3; N, 32·35 per cent). It yielded a sparingly soluble yellow hydrochloride with dilute hydrochloric acid.

2,4-*Diamino-6-morpholino-5(4'-nitrophenylazo)pyrimidine* (CB. 2270, No. 22)

2:4-Diamino-6-chloro-5(4'-nitrophenylazo)pyrimidine (1 g.) was treated with dry morpholine (10 ml.) when the mixture became warm. The reaction was completed by heating under reflux for 10 minutes, and on cooling the mixture deposited red crystals. These were collected, washed with cold ethanol and recrystallised from aqueous 2-ethoxyethanol to yield the *morpholino* compound as deep red prisms, m.p. 275° (decomp.). (Found C, 49·2; H, 4·60; N, 32·5. $C_{14}H_{16}O_3N_8$ requires C, 48·9; H, 4·65; N, 32·55 per cent). The compound had a pronounced golden-green metallic lustre.

2:4-*Diamino-6-diethanolamino-5(4'-nitrophenylazo)pyrimidine* (CB. 2271, No. 23)

This was prepared similarly from the azo compound (1 g.) and dry diethanolamine (5 ml.). The *diethanolamino* compound, obtained by dilution of the reaction mixture with water, crystallised from very dilute ethanol as deep maroon plates, with a metallic lustre, m.p. 211 to 212°

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to Cavalieri and others[14], and 8-azapurine (No. 64), made by ring-closure of 4:5-diaminopyrimidine, were synthesised to ascertain the effect on activity of removing successively the aryl substituent and the amino groups from the diamino-8-azapurines. Both were inactive.

T: (No. 68) and 2·4:5:6-tetra- y Traube's method, for antifolic ucts of the diamino-hydroxy and triamino-arylazopyrimidines respectively. Since both types of azo-pyrimidine are active, the fact that V (R' = OH)[15] and V (R' = NH₂)[16] are respectively inactive and active, argues against the hypothesis that they might be the active forms.

New compounds are indicated in the experimental section by italics and, in the Tables, by the absence of references to their preparation.

EXPERIMENTAL

Melting points were determined using an electrically heated copper block and are uncorrected unless otherwise stated. Samples for analysis were dried at 100° in a high vacuum unless otherwise stated. Analyses were by Drs. Weiler and Strauss (Oxford) and by Mr. P. R. W. Baker (Beckenham).

2:4-*Diamino*-6-*chloro*-5(4'-*nitrophenylazo*)-*pyrimidine* (CB. 2297, No. 19)

2:4-Diamino-6-chloropyrimidine (1·44 g.) in dilute acetic acid (3N, 75 ml.) was cooled in ice-water and stirred and then treated with a solution of p-nitrobenzenediazonium chloride (from p-nitraniline (1·38 g.)), freed from nitrous acid by treatment with excess of sulphamic acid. A slight change in colour was noted. After stirring the solution for 5 minutes, it was treated with crystalline sodium acetate to bring the pH to 6–7 and stirring was continued for 5 hours, during which time a thick orange-red precipitate was obtained. This was collected, washed with water copiously, dried and recrystallised from aqueous 2-ethoxyethanol to yield the *azo-compound* as scarlet needles, m.p. 298 to 299° (decomp.) (Found: C, 38·7; H, 3·3; N, 31·4; Cl, 11·3. $C_{10}H_8O_2N_7Cl.1H_2O$ requires C, 38·5; H, 3·2; N, 31·5; Cl, 11·4. Found on a sample dried at 110°: C, 41·15; H, 2·9; loss 5·8. $C_{10}H_8O_2N_7Cl$ requires C, 40 9; H, 2·7; loss, 5·8 per cent).

2:4-*Diamino*-6-*chloro*-5(4'-*chlorophenylazo*)*pyrimidine* (CB. 2298, No. 20)

2:4-Diamino-6-chloropyrimidine (1·44 g.) in dilute acetic acid (3N, 75 ml.) was treated with p-chlorobenzenediazonium chloride (from p-chloroaniline (1·275 g.)) and the product isolated as above. Recrystallised from aqueous 2-ethoxyethanol, the *azo-compound* formed fine yellow needles m.p. 271 to 272° (decomp.). (Found: C, 42·1; H, 3·1; N, 29·3; Cl, 25·0. $C_{10}H_8N_6Cl_2$ requires C, 42·4; H, 2·8; N, 29·7; Cl, 25·1 per cent).

2:4-*Diamino*-6-*dimethylamino*-5(4'-*nitrophenylazo*)-*pyrimidine* (CB. 2269, No. 21)

Method 1. 2:4-Diamino-6-chloro-5(4'-nitrophenylazo)-pyrimidine (1 g.) in dry 2-ethoxyethanol (40 ml.) was treated with an ethanolic solution

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2:4-Diamino-5-(2'-chlorophenylazo)-6-diethanolaminopyrimidine (CB. 2280, No. 26)

The chlorophenylazo-6-chloropyrimidine 7·1 g., and diethanolamine 5 mL.) were heated together at 140° for 2 hours, cooled and diluted with water. The resulting oily gum crystallised on being allowed to stand overnight and the solid, after crystallisation from very dilute ethanol, yielded the diethanolamino compound as orange brown prisms, m.p. 150 to 152°. (Found: C, 48·9; H, 5·0; N, 24·5; C, 9·4. $C_{14}H_{19}O_2N_6Cl$ requires C, 47·4; H, 5·2; N, 22·4; Cl, 9·6 per cent.

2:4:6-Triamino-5-arylazopyrimidines

General Method. 2:4:6-Triaminopyrimidine 5·4 g., 0·25 mol.) was dissolved in water (150 mL.) containing crystalline sodium acetate (75 g.) and the mixture stirred mechanically at 0° during the addition of a filtered solution of the required diazotised amine. The latter solution was generally prepared by dissolving the arylamine (0·25 mol.) in a mixture of concentrated hydrochloric acid (70 mL.) and water (70 mL.), cooling to 0° and adding a solution of sodium nitrite 2·14 g.) in water (70 mL.); after 2–3 minutes, an excess of solid sulphamic acid was added and the solution filtered rapidly through a chilled funnel under suction. Modification of the procedure was necessary only if the amine was not easily basic to be soluble in dilute hydrochloric acid. An extreme example was 2:4:6:6-tribromoaniline, which was diazotised as follows. The amine (6·6 g., 0·50 mol.) was dissolved in hot glacial acetic acid (70 mL.) and crystallised on cooling. The suspension of small crystals was added to a solution of sodium nitrite (1·54 g.) in cold concentrated sulphuric acid (70 mL.) and gave a pale yellow solution. This cooled rapidly on addition to a solution of triaminopyrimidine 0·5 g., in water 5% ml.) and the product was coloured after adjustment of the pH of the solution to 6–7 by the addition of sodium acetate crystals.

Occasionally, despite the presence of sodium acetate in the solution before addition of the diazonium salt, the azo compound was precipitated as the hydrochloride. This could be converted to the base by boiling with aqueous pyridine.

The azo compounds so obtained are listed in Table 1.

Other 4-amino-5-arylazopyrimidines

2:4-Diamino-5-(4-nitrophenylazo)-6-hydroxypyrimidine (CB. 1201, No. 27)

2:4-Diamino-6-hydroxypyrimidine hydrochloride (6·5 g., was suspended in water (360 mL.) mixed with sodium acetate 40 g. and thereto a solution of p-nitrobenzene diazonium chloride from p-chloroaniline (50 g.) was slowly added to the ice-cold filtrate with stirring and the orange-yellow precipitate was collected after two hours. It was crystallised from aqueous pyridine when a pyridine salt was formed, which was decomposed by stirring with hot ethanol to liberate the azo compound as a yellow microcrystalline powder, m.p. 294 to 305° (decomp.). Found:

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(decomp.). (Found: C, 46·2; H, 5·1; N, 30·7. $C_{14}H_{18}O_4N_8$ requires C, 46·4; H, 5·0; N, 30·9 per cent). It was very soluble in ethanol.

2:4-Diamino-5(4'-chlorophenylazo)-6-dimethylamino pyrimidine (CB. 2266, No. 24)

Method 1. 2:4-Diamino-6-chloro-5(4'-chlorophenylazo) pyrimidine (1 g.) in dry 2-ethoxyethanol (40 ml.) was heated under reflux for one hour with an ethanolic solution of dimethylamine (33 per cent, 3 ml.). The clear solution was then filtered and concentrated, and on dilution then yielded a yellow precipitate. This on recrystallisation from aqueous 2-ethoxyethanol yielded the *dimethylamino* compound (0·6 g.) as long lustrous yellow needles, m.p. 210° (corr.), undepressed on admixture with the product from Method 2.

Method 2. 2:4-Diamino-6-chloro-5(4'-chlorophenylazo)pyrimidine (0·5 g.) was dissolved in dimethylformamide (10 ml.) and treated with a solution of guanidine in ethanol (from guanidine hydrochloride (0·675 g.) and a solution of sodium (0·16 g.) in ethanol (5 ml.), with removal of sodium chloride). The mixture was heated under reflux at 60 to 70° for 4 hours and then the solvent was removed under reduced pressure. Addition of water yielded a yellow solid which was crystallised from aqueous 2-ethoxyethanol to yield the 6-*dimethylamino* compound as yellow needles, m.p. 210° (corr.) or lustrous golden leaflets, m.p. 187 to 188° depending on the rate of cooling. (Found: C, 49·3; H, 4·9; N, 33·75; Cl, 12·3. $C_{12}H_{14}N_7Cl$ requires C, 49·4; H, 4·8; N, 33·6; Cl, 12·2 per cent).

Careful search failed to reveal a second component (cf. above).

2:4-Diamino-5(4'-chlorophenylazo-6(β-ethoxyethoxy))pyrimidine

2:4-Diamino-6-chloro-5(4'-chlorophenylazo)pyrimidine (0·5 g.) was added to a solution of guanidine (from guanidine hydrochloride (0·675 g.) and sodium (0·16 g.) dissolved in 2-ethoxyethanol (20 ml.) with removal of the sodium chloride) and the mixture heated under reflux at 80° for three hours. Concentration of the filtered solution under reduced pressure at 100° and dilution of the residue with water yielded a yellow solid which on recrystallisation from aqueous 2-ethoxy ethanol (90 per cent) afforded the *azopyrimidine* as yellow lustrous silky needles, m.p. 161 to 162°. (Found C, 50·1; H, 5·0; N, 25·1; Cl, 10·6. $C_{14}H_{17}O_2N_6Cl$ requires C, 49·95; H, 5·05; N, 25·0; Cl, 10·55 per cent).

2:4-Diamino-5(4'-chlorophenylazo)-6-morpholinopyrimidine (CB. 2279, No. 25)

The chlorophenylazo-6-chloropyrimidine (0·3 g.) and morpholine (2 ml.) were heated together under reflux for one hour, cooled and diluted with water to yield a yellow precipitate. This, on crystallisation from aqueous 2-ethoxyethanol, afforded the *morpholino pyrimidine* as golden-yellow lustrous plates, m.p. 221 to 222°. (Found C, 50·7; H, 4·7, N, 28·9; Cl, 10·5 $C_{14}H_{16}ON_7Cl$ requires C, 50·4; H, 4·8; N, 29·4; Cl, 10·65 per cent).

2 : 4-*Diamino*-5(4'-*chlorophenylazo*)-6-*diethanolaminopyrimidine* (CB. 2280, No. 26)

The . . . . imidine (1·0 g.) and diethanolamine (5 ml.) . . . . 2 hours, cooled and diluted with water. The resulting oily ب. . . . . llised on being allowed to stand overnight and the solid, after crystallisation from very dilute ethanol, yielded the *diethanolamino* compound as orange brown prisms, m.p. 160 to 161°. (Found: C, 48·15; H, 5·12; N, 28·25; Cl, 10·4. $C_{14}H_{18}O_2N_7Cl$ requires C, 47·8; H, 5·12; N, 27·9; Cl, 10·1 per cent).

2 : 4 : 6-*Triamino*-5-*arylazopyrimidines*

. . . . . . 2-. . . . . -line (5·0 g., 1/25 mol.) was

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solution of the requ. . . . . . . • . . . .

ally prepared by dissolving the aryl. . . . . re of concentrated hydrochloric acid (12 ml.) and water (20 ml.), co. . ng to 0° and adding a solution of sodium nitrite (3·04 g.) *in* water (20 ml.); after 2–3 minutes, an excess of solid sulphamic acid was added and the solution filtered rapidly *through* a chilled funnel under *suction*. Modification of the procedure was necessary only if the amine was too weakly basic to be soluble in dilute hydrochloric acid. An extreme example was 2 : 4 : 6-tribromoaniline, which was diazotised as follows. The amine (6·6 g., 1/50 mol.) was dissolved in hot glacial acetic acid (79 ml.) and crystallised on cooling. The suspension of small crystals was added to a solution of sodium nitrite (1·54 g.) in cold concentrated sulphuric acid (10 ml.) and gave a pale yellow solution. This coupled normally on addition to a solution of triaminopyrimidine (2·5 g.) in water (500 ml.) and the product was collected after adjustment of the pH of the solution to 6–7 by the addition of sodium acetate crystals.

Occasionally, despite the presence of sodium acetate in the solution before addition of the diazonium salt, the azo compound was precipitated as the hydrochloride. This could be converted to the base by boiling with aqueous pyridine.

The azo compounds so obtained are listed in Table I.

Other 4-amino-5-arylazopyrimidines

2 : 4-*Diamino*-5(4'-*chlorophenylazo*)-6-*hydroxypyrimidine* (CB. 2288, No. 27)

2 . 4-Diamino-6-hydroxypyrimidine hydrochloride (6·5 g.) was suspended in water (360 ml.) stirred with sodium acetate (42 g.) and filtered. A solution of *p*-chlorobenzene diazonium chloride (from *p*-chloroaniline (5·1 g.)) was slowly added to the ice-cold filtrate with stirring and the orange-yellow precipitate was collected after two hours. It was crystallised from aqueous pyridine when a pyridine salt was obtained, which was decomposed by stirring with hot ethanol to liberate the *azo-compound* as a yellow microcrystalline powder, m.p. 314 to 315° (decomp.). (Found:

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TABLE I

ARYL-AZOPYRIMIDINES (III, R' = R'' = NH₂)

Cpd. No.	R	Colour and crystal form	M.p. °	Solvent	Formula	Found					Required				
						C	H	N	Cl	Br	C	H	N	Cl	Br
*2295 No 1	C_6H_5-	Yellow leaflets	262–263	CW	$C_{10}H_{10}N_6$	52.5	4.6	42.4	—	—	52.4	4.8	42.8	—	—
2110 No 2	2-ClC_6H_4-	Yellow needles	290–291	PW	$C_{10}H_9N_6Cl$	45.7	3.9	36.6	13.6	—	45.5	3.8	37.2	13.5	—
2309 No 3	3-ClC_6H_4-	Yellow needles	264–265	PW	''	45.7	3.7	37.0	13.6	—	"	"	"	"	—
2177 No 4	4-ClC_6H_4-	Golden-yellow flat needles	262	CW	''	45.9	4.2	36.7	13.3	—	"	"	"	"	—
2128 No 5	2-BrC_6H_4-	Golden-yellow crystals	298–299	CW	$C_{10}H_9N_6Br$	39.5	3.3	31.9	—	26.0	39.0	3.25	31.8	—	26.0
2311 No 6	4-BrC_6H_4-	Yellow prisms	261–262	CW	''	39.0	3.3	31.7	—	25.7	40.3	3.0	32.9	—	"
2126 No 7	2:3-$Cl_2C_6H_3-$	Orange-yellow leaflets	316–317	CW	$C_{10}H_8N_6Cl_2$	40.6	2.9	32.7	23.9	—	40.3	3.0	32.9	23.8	—
2307 No 8	2.4-$Cl_2C_6H_3-$	Long yellow leaflets	304–305 (decomp.)	CW	''	40.3	3.0	32.5	24.1	—	"	"	"	"	—
2322 No 9	2:5-$Cl_2C_6H_3-$	Yellow needles	319–320	PW	''	40.4	3.0	32.7	23.6	—	"	"	"	"	—
2283 No 10	3:4-$Cl_2C_6H_3-$	Yellow leaflets	269–270	PW	''	40.4	3.0	32.6	23.7	—	"	"	"	"	—
2349 No 11	2.4-$Br_2C_6H_3-$	Orange-yellow leaflets	328–329	PW	$C_{10}H_8N_6Br_2$	31.3	2.5	25.3	—	41.3	31.0	2.3	25.3	—	41.4
2347 No 12	2.4:6-$Br_3C_6H_2-$	Orange-yellow needles	304–305 (decomp)	CW	$C_{10}H_7N_6Br_3$	25.8	2.0	20.6	—	51.4	25.75	1.7	21.05	—	51.5
2299 No 13	4-$MeOC_6H_4-$	Orange-yellow prisms	231–232	CW	$C_{11}H_{12}ON_6$	50.8	5.4	37.7	—	—	51.0	5.0	37.8	—	—
2323 No 14	4-$EtOC_6H_4-$	Yellow needles	280–282	CW	$C_{12}H_{14}ON_6$	53.1	5.3	33.7	—	—	52.8	5.5	35.9	—	—
†2296 No 15	4-$NO_2C_6H_4-$	Red needles	352–353	ACOH	$C_{10}H_9O_2N_7$	44.1	3.5	40.7	—	—	43.8	3.65	40.9	—	—
2110 No 16	1-naphthyl	Golden-brown plates	264–265 (decomp)	CW	$C_{14}H_{12}N_6$	60.6	4.6	35.3	—	—	60.2	4.7	35·15	—	—
2315 No 17	2-naphthyl	Yellow needles	298 (decomp)	B	''	59.9	4.4	34.9	—	—	"	"	"	—	—
2311 No 18	3-quinolyl	Orange leaflets, blue lustre	315–316 (decomp)	PW	$C_{13}H_{11}N_7$	56.0	4.5	39.9	—	—	55.7	4.3	40.0	—	—

* First prepared by Hartzel and Benson (*J. Amer. chem. Soc.*, 1954, 76, 2263) who gave m.p. 215° (decomp.).
† Yellow acetate, initially isolated, is decomposed either on standing or on washing with ethanol and ether.
‡ Solvents: B, *n*-butanol; CW, aqueous 2-ethoxyethanol; PW, aqueous pyridine; ACOH, glacial acetic acid

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C, 45·5; H, 3·5; N, 31·5; Cl, 13·2. $C_{10}H_9ON_6Cl$ requires C, 45·4; H, 3·4; N, 31·73; Cl, 13·4 per cent).

2:4-*Diamino*-5(4'-*bromophenylazo*)-6-*hydroxypyrimidine* (CB. 2336, No. 28)

This was obtained in a similar manner when p-bromobenzene-diazonium chloride was used. The *azo-compound* was purified by successive crystallisations from aqueous pyridine and aqueous 2-ethoxyethanol and formed yellow crystals. (Found: C, 38·9; H, 3·5; N, 27·3; Br, 26·0. $C_{10}H_9ON_6Br$ requires C, 38·9; H, 3·0; N, 27·2; Br, 25·9 per cent).

4-*Amino*-5(4'-*chlorophenylazo*)-2:6-*dihydroxypyrimidine* (CB. 2313, No. 29)

4-Amino-2:6-dihydroxypyrimidine (5·1 g.) was suspended in water and sufficient 2N sodium hydroxide was added dropwise to yield a clear solution. This solution was then cooled in ice, stirred and treated with a filtered solution of p-chlorobenzene diazonium chloride (from p-chloroaniline (5·1 g.)). Glacial acetic acid was added dropwise to the mixture to adjust the pH to 5–6 and after the mixture had remained at 0° in the ice-chest overnight, the product was collected. It was purified by crystallisation from hot 90 per cent formic acid by the addition of water and the resulting yellow solid was dissolved in hot 6N ammonium hydroxide, the solution filtered and the product reprecipitated by neutralisation using glacial acetic acetic acid. The *azo-compound* formed a fine yellow powder which was thoroughly washed by means of water, ethanol and ether successively. (Found: C, 45·15; H, 2·9; N, 26·2; Cl, 13·2; $C_{10}H_8O_2N_5Cl$ requires C, 45·2; H, 3·0; N, 26·4; Cl, 13·4 per cent).

2:4-*Diamino*-5(4'-*chlorophenylazo*)*pyrimidine* (CB. 2329, No. 30)

This was prepared as described by Brown[17]. It formed yellow needles from aqueous 2-ethoxyethanol, m.p. 282 to 283° (decomp.). Brown (*loc. cit.*) gives m.p. 271 to 272°.

4-*Amino*-5(4'-*chlorophenylazo*)-6-*hydroxypyrimidine* (CB. 2304, No. 31)

A solution of 4-chlorobenzenediazonium chloride (from p-chloroaniline (10·2 g.)) was added slowly with stirring to a filtered, ice-cold solution of 4-amino-6-hydroxypyrimidine (8·82 g.) in water (850 ml.). The pH of the reaction mixture was adjusted to 5–6 by the addition of crystalline sodium acetate and the product collected after a period. Recrystallisation from aqueous 2-ethoxyethanol afforded the *azo-compound* as orange needles, m.p. 300°. (Found: C, 48·6; H, 3·3; N, 27·8; Cl, 14·1. $C_{10}H_8ON_5Cl$ requires C, 48·1; H, 3·2; N, 28·1; Cl, 14·2 per cent).

4:6-*Diamino*-5(4'-*chlorophenylazo*)-2-*thiopyrimidine* (CB. 2292, No. 32) and 4:6-*Diamino*-5(4'-*chlorophenylazo*)-2(4'-*chlorophenylthio*) *pyrimidine* (CB. 2290, No. 33)

A solution of 4-chlorobenzenediazonium chloride (from p-chloroaniline (5·1 g.)) was added slowly to an ice-cold, stirred solution of 4:6-diamino-2-thiopyrimidine (5·68 g.) in dilute hydrochloric acid (0·1N, 500 ml.).

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On adjusting the pH to 5–6 by the addition of crystalline sodium acetate, a slow precipitation of yellow solid was observed. This was completed after the mixture had stood overnight at 0°. The product was extracted with boiling ethanol and the hot filtrate diluted with water to yield 4:6-*diamino-5(4'-chlorophenylazo)-2-(4'-chlorophenylthio) pyrimidine* as yellow feathery needles from aqueous ethanol, m.p. 211 to 212°. (Found: C, 49·3; H, 3·0; N, 21·35; S, 8·35; Cl, 17·6. $C_{16}H_{12}N_6SCl_2$ requires C, 49·1; H, 3·1; N, 21·5; S, 8·2; Cl, 18·2 per cent).

The residue from the ethanol extraction was crystallised several times from aqueous pyridine and afforded 4:6-diamino-5 (4'-chlorophenylazo)-2-thiopyrimidine as yellow needles, m.p. 278 to 279° (decomp.). Found: C, 43·2; H, 3·1; N, 29·7; S, 11·3; Cl, 13·0. $C_{10}H_9N_6SCl$ requires C, 42·8; H, 3·2; N, 30·0; S, 11·4; Cl, 12·7 per cent).

4:6-Diamino-5(4'-chlorophenylazo)pyrimidine (CB. 2287, No. 34)·

4:6-Diamino pyrimidine (4·4 g.) was dissolved in dilute hydrochloric acid (500 ml. 0 04N) and coupled by treating it with a filtered solution of 4-chlorobenzene diazonium chloride (from p-chloroaniline (5·1 g.)) in the usual manner. The addition of solid sodium acetate to the reaction mixture afforded a precipitate which was collected and recrystallised from aqueous 2-ethoxyethanol when the azo compound was obtained as flat yellow needles, m.p. 299 to 300° (decomp.). (Found: C, 48·6; H, 3·5; N, 34·0; Cl, 14·25. Calc. for $C_{10}H_9N_6Cl$: C, 48·3; H, 3·6; N, 33·85; Cl, 14·3 per cent). Lythgoe, Todd and Topham[18] give m.p. 301 to 302° (decomp.).

The preparation of 2:6-diamino-3(4'-chlorophenylazo)pyridine is given at the end of the experimental section.

8-Aryl-8-azapurines

With the exception of the few members noted below, which include a 1-deaza-8-azapurine (No. 60), the compounds of this series were all prepared in the following general manner.

General Method of preparation of 8-aryl-azapurines

The corresponding azopyrimidine (4 g.) was dissolved in a mixture of pyridine (100 ml.) and water (100 ml.) containing copper sulphate (10 g.). When the azo compound was insoluble in this mixture either a larger proportion of pyridine was used, e.g., 200 ml. pyridine to 100 ml. of water, or else a larger volume of mixed solvents in 1 : 1 proportion. The mixture was placed in a three-necked flask equipped with reflux condenser and a gas inlet tube reaching to the bottom of the flask, and heated to boiling under reflux. A slow stream of oxygen from a cylinder was then passed until the reaction was complete, which was indicated when the solution changed from a greenish-yellow hue to a rich royal blue colour. The hot solution was then poured into water (2 litres) and allowed to stand over-night to ensure complete precipitation. The 8-azapurine was then collected at the pump, washed with copious quantities of water and then with ethanol and finally ether. In nearly every case the yield was quantitative.

TABLE II
8-ARYL-8-AZAPURINES (IV)

CB No.	R	R'	R''	Formula	M.p. °	Solvent§	Found C	Found H	Found N	Found Cl	Found Br	Found S	Req. C	Req. H	Req. N	Req. Cl	Req. Br	Req. S
*[2]214 No 33	C6H5–	NH2	NH2	C9H7N5	344–345 (decomp.)	20 F	—	—	—	—	—	—	45.9	3.1	37.5	—	—	—
2321 No. 36	2-ClC6H4–	NH2	NH2	C9H6N5Cl	283–284	20 F	45.9	3.2	37.7	13.3	—	—	45.9	3.1	37.5	13.6	—	—
2209 No. 37	3-ClC6H4–	NH2	NH2	"	330 (decomp.)	20 F	46.2	3.0	37.2	13.8	—	—	46.2	"	"	"	—	—
2278 No 38	4-ClC6H4–	NH2	NH2	"	>360	20 F	46.3	2.9	37.3	13.4	—	—	46.2	"	"	"	—	—
2332 No. 39	2-BrC6H4–	NH2	NH2	C9H6N5Br	211–212	10 F	37.65	2.8	27.5	—	23.2	—	37.5	2.8	27.83	—	22.7	—
2319 No. 40	4-BrC6H4–	NH2	NH2	C9H6N5Br·½HCO2H	>360	20 F	39.1	3.0	30.6	—	24.5	—	39.5	2.7	32.9	—	24.3	—
2333 No 41	2,3-Cl2C6H3–	NH2	NH2	C9H5N5Cl2·½HCO2H	287–288	20 F	39.35	3.25	30.6	22.7	—	—	39.5	2.5	33.1	22.3	—	—
2318 No 42	2,4-Cl2C6H3–	NH2	NH2	C9H5N5Cl2	320–321	20 F	40.1	3.4	32.5	23.85	—	—	40.55	2.4	33.1	24.0	—	—
2324 No 43	2,5-Cl2C6H3–	NH2	NH2	C9H5N5Cl2	312–313	20 F	40.7	2.5	32.9	23.8	—	—	40.55	2.4	33.1	24.0	—	—
2282 No. 44	3,4-Cl2C6H3–	NH2	NH2	C9H5N5Cl2	>360	20 F	40.0	2.5	32.9	24.2	—	—	40.55	2.4	33.1	24.0	—	—
2364 No. 45	2,4-Br2C6H3–	NH2	NH2	C9H5N5Br2·½HCO2H	279–281 (decomp.)	20 F	30.6	2.5	25.5	—	37.0	—	30.65	2.1	22.75	—	37.15	—
2330 No 46	2,4,6-Br3C6H2–	NH2	NH2	C9H4N5Br3	286–287	AW	26.1	2.5	12.09	—	51.3	—	25.9	1.3	11.1	—	51.7	—
2327 No. 47	4-EtO-C6H4–	NH2	NH2	C11H11ON5·½HCO2H	310–311	20 F	51.7	4.4	34.5	—	—	—	52.0	4.8	34.7	—	—	—
2335 No. 48	1-C10H7–	NH2	NH2	C13H9N5	290–291	20 F	60.4	3.7	35.2	—	—	—	60.7	3.9	35.4	—	—	—
*2239 No. 49	4-ClC6H4–	NH2	OH	C9H5ON4Cl	>360	89 E	43.7	3.1	31.9	13.2	—	—	43.8	2.7	32.2	13.5	—	—
2337 No. 50	4-BrC6H4–	OH	NH2	C9H5ON4Br	>360	89 E	39.0	2.2	27.3	—	26.6	—	39.1	2.3	27.4	—	26.1	—
2285 No 51	4-ClC6H4–	NMe2	NH2	C11H11N5Cl·HCl	287–288	2N HCl	44.5	3.8	29.9	21.6	—	—	44.2	3.9	30.05	21.8	—	—
2293 No 52	4-NO2C6H4–	NMe2	NH2	C11H12O2N6	316–318	DMF	48.1	3.7	37.0	—	—	—	48.0	4.0	30.05	—	—	—
2291 No. 53	4-ClC6H4–	NH2	4-ClC6H5	C15H9N5Cl2	325–326 (decomp.)	80 F	49.3	3.27	21.25	17.9	—	—	49.4	2.6	21.6	18.3	—	8.2
2313 No. 54	4-ClC6H4–	NH2	R	C9H8N5Cl·HCl	367 (decomp.)	ACOH	49.1	3.1	31.1	14.0	—	—	48.7	2.8	34.1	14.4	—	—
2205 No 55	4-ClC6H4–	OH	H	C9H5ClON4	339–340	CW	48.7	2.5	28.5	14.1	—	—	48.5	2.6	28.3	14.3	—	—

* Parker and Webb, U.S.P. 2,543,333; Chem. Abstr. 1951, 45, 7603
† Hertel and Bension (J. Amer. chem. Soc. 1954, 76, 2263) give m.p. >300°.
‡ No preparation described in literature; compounds mentioned in Stock and others (Cancer Research, Suppl. 1, p 104 as compound No 1189 (m.p. 375°), obtained from Calco Division, American Cyanamid Co.
§ Solvents: F, Aqueous formic acid, percentage content indicated; DMF, Dimethylformamide; ACOH, Glacial acetic acid; CW, Aqueous 2-ethoxyethanol; AW, Aqueous ethanol.

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On adjusting the pH to 5–6 by the addition of crystalline sodium acetate, a slow precipitation of yellow solid was observed. This was completed after the mixture had stood overnight at 0°. The product was extracted with boiling ethanol and the hot filtrate diluted with water to yield 4:6-*diamino-5(4'-chlorophenylazo)-2-(4'-chlorophenylthio) pyrimidine* as yellow feathery needles from aqueous ethanol, m.p. 211 to 212°. (Found: C, 49·3; H, 3·0; N, 21·35; S, 8·35; Cl, 17·6. $C_{16}H_{12}N_6SCl_2$ requires C, 49·1; H, 3·1; N, 21·5; S, 8·2; Cl, 18·2 per cent.)

The residue from the ethanol extraction was crystallised several times from aqueous pyridine and afforded 4:6-diamino-5 (4'-chlorophenylazo)-2-thiopyrimidine as yellow needles, m.p. 278 to 279° (decomp.). Found: C, 43·2; H, 3·1; N, 29·7; S, 11·3; Cl, 13·0. $C_{10}H_9N_6SCl$ requires C, 42·8; H, 3·2; N, 30·0; S, 11·4; Cl, 12·7 per cent).

4:6-*Diamino-5(4'-chlorophenylazo)pyrimidine* (CB. 2287, No. 34)·

4:6-Diamino pyrimidine (4·4 g.) was dissolved in dilute hydrochloric acid (500 ml. 0·04N) and coupled by treating it with a filtered solution of 4-chlorobenzene diazonium chloride (from *p*-chloroaniline (5·1 g.)) in the usual manner. The addition of solid sodium acetate to the reaction mixture afforded a precipitate which was collected and recrystallised from aqueous 2-ethoxyethanol when the azo compound was obtained as flat yellow needles, m.p. 299 to 300° (decomp.). (Found: C, 48·6; H, 3·5; N, 34·0; Cl, 14·25. Calc. for $C_{10}H_9N_6Cl$: C, 48·3; H, 3·6; N, 33·85; Cl, 14·3 per cent). Lythgoe, Todd and Topham[18] give m.p. 301 to 302° (decomp.).

The preparation of 2·6-diamino-3(4'-chlorophenylazo)pyridine is given at the end of the experimental section.

8-*Aryl-8-azapurines*

With the exception of the few members noted below, which include a 1-deaza-8-azapurine (No. 60), the compounds of this series were all prepared in the following general method.

General Method of preparation of 8-aryl-azapurines

The corresponding azopyrimidine (4 g.) was dissolved in a mixture of pyridine (100 ml.) and water (100 ml.) containing copper sulphate (10 g.). When the azo compound was insoluble in this mixture either a larger proportion of pyridine was used, e.g., 200 ml. pyridine to 100 ml. of water, or else a larger volume of mixed solvents in 1:1 proportion. The mixture was placed in a three-necked flask equipped with reflux condenser and a gas inlet tube reaching to the bottom of the flask, and heated to boiling under reflux. A slow stream of oxygen from a cylinder was then passed until the reaction was complete, which was indicated when the solution changed from a greenish-yellow hue to a rich royal blue colour. The hot solution was then poured into water (2 litres) and allowed to stand overnight to ensure complete precipitation. The 8-azapurine was then collected at the pump, washed with copious quantities of water and then with ethanol and finally ether. In nearly every case the yield was quantitative.

Besides being soluble in dilute acid, the compound was soluble in hot dilute sodium hydroxide, revealing the presence of a hydroxy group.

2.6-Diamino-8-(3'-quinolyl)-8-azapurine (CB. 2381, No. 59)

The ring closure of triamino-5(3'-quinolylazo)pyrimidine was carried out exactly as described above. The crude product was recrystallised from propylene glycol (charcoal) several times, washed thoroughly by trituration with ethanol and finally ether and yielded a *pale cream solid*, m.p. > 360°, which however, was now not soluble in hot dilute sodium hydroxide. To obtain satisfactory analytical figures, it was necessary to dry the sample to constant weight at 140°. (Found: C, 56·3; H, 4·0; N, 39·9; $C_{13}H_{10}N_8$ requires C, 56·2; H, 3·6; N, 40·3 per cent).

5-Amino-2(4'-chlorophenyl)-triazolo(4':5'-2:3)pyridine (CB. 2363, No. 60)

2:6-Diamino-3(4'-chlorophenylazo)pyridine (5 g.) was dissolved in a mixture of pyridine (100 ml.) and water (100 ml.) containing copper sulphate pentahydrate (10 g.) and was heated under reflux during the passage of oxygen. A rapid reaction occurred and a deep blue colour developed within 10 minutes. After one hour, the mixture was poured into water (2 l.) and the pale grey precipitate was collected. Crystallisation from aqueous 2-ethoxyethanol gave the *triazolopyridine* as long white felted needles, changing on standing to pale yellow prismatic needles. The change could be accelerated by warming; both forms melted at 258 to 259°. (Found. C, 54·1; H, 3·2; N, 28·65; Cl, 14·1. $C_{11}H_8N_5Cl$ requires C, 53·8; H, 3·3; N, 28·5; Cl, 14·5 per cent.) Treatment of this compound with hot acetic anhydride afforded the *5-acetamido* derivative as colourless needles, m.p. 296 to 297° from *n*-butanol. (Found. C, 53·0; H, 3·5; N, 23·2; Cl, 12·2. $C_{13}H_{10}ON_5Cl.\frac{1}{2}H_2O$ requires C, 52·6; H, 3·5; N, 23·6; Cl, 12·0 per cent).

9-Aryl-8-azapurines

A. 2-Amino-9-phenyl-8-azapurine

(1) *2-Amino-4-anilino-5-nitropyrimidine*.   4-Anilino-2-chloro-5-nitropyrimidine (3·0 g.)[19] was heated for four hours at 100° in a sealed tube with saturated ethanolic ammonia (25 ml.). The tube was cooled, opened and the lemon yellow product (2·91 g.) collected and crystallised from *n*-butanol to give the *aminonitropyrimidine* as lemon-yellow needles, m.p. 206 to 207°. (Found: C, 52·3, H, 3·9, N, 30·05. $C_{10}H_9O_2N_5$ requires C, 52·0; H, 3·9, N, 30·3 per cent).

(ii) *2·5-Diamino-4-anilino pyrimidine*. The above aminonitropyrimidine (1·8 g.) was stirred into a solution of stannous chloride (7·6 g.) in concentrated hydrochloric acid (38 ml.) and the mixture was gently warmed on the steam bath. When all the yellow solid had been replaced by a *white precipitate*, the mixture was cooled and the solid collected and dissolved in water. The solution was treated with 40 per cent sodium hydroxide solution and the white precipitate collected using chloroform. Evaporation of the dried extract gave a pale yellow residue which was

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Table II lists the compounds prepared in this way. All were colourless solids, generally of microcrystalline form. The majority could only be crystallised from aqueous formic acid and occasionally retained formic acid tenaciously after prolonged drying at 100°. In every case the acid content was confirmed by drying at a higher temperature and recording the required loss, the product then analysed for carbon and hydrogen with satisfactory results. It is not possible to show this in the Table.

8-(4'-Chlorophenyl)-6-thio-8-azapurine (CB. 2320, No. 56)

8-(4'-Chlorophenyl)6-hydroxy-8-azapurine (3 g.) in dry pyridine (50 ml.) was treated with phosphorus pentasulphide (3 g.) and the mixture was heated under reflux for one and a half hours, all material dissolving during the initial half hour to yield a dark-brown solution. The reaction mixture was allowed to cool somewhat and was then smoothly decomposed by adding it to hot water (100 ml.). The product (2·87 g.) was collected next day and washed thoroughly with water and then with ethanol and ether. Two crystallisations from aqueous pyridine (charcoal) afforded the *thio-compound* as pale golden lustrous needles, m.p. 357 to 358° (decomp.). (Found: C, 46·0; H, 2·5; N, 26·3; S, 12·1; Cl, 13·4. $C_{10}H_6N_5SCl$ requires C, 45·5; H, 2·3; N, 26·6; S, 12·15; Cl, 13·5 per cent).

2-Amino-8(4'-Chlorophenyl)-6-thio-8-azapurine (CB. 2294, No. 57)

A mixture of 2-amino-8(4'-chlorophenyl)-6-hydroxy-8-azapurine (1 g.) and phosphorus pentasulphide (1 g.) in dry pyridine (50 ml.) was heated under reflux for two hours. A further portion of phosphorus pentasulphide (2 g.) and pyridine (50 ml.) was then added and heating continued. All material had dissolved after a further hour and after a total time of reflux of 4 hours, the mixture was decomposed by pouring into hot water (200 ml.). The primrose-yellow precipitate (Crude 0·91 g.) which was formed was collected next day and crystallised from pyridine (charcoal) to yield the *thio compound* as a yellow powder, m.p. 362 to 363° (decomp.). (Found: C, 45·3; H, 3·0; N, 28·9; S, 10·4; Cl, 12·1. $C_{10}H_7N_6SCl$-1/4 C_5H_5N requires C, 45·3; H, 2·8; N, 29·3; S, 10·7; Cl, 11·9 per cent).

2-Amino-6-hydroxy-8(3'-quinolyl)-8-azapurine (CB. 2338, No. 58)

2:4:6-Triamino-5(3'-quinolylazo) pyrimidine (12·0 g.) in pyridine (600 ml.) and water (100 ml.) was treated with a solution of copper sulphate pentahydrate (40 g.) in hot water (100 ml.) and the clear solution was heated in a three-necked flask fitted with reflux condenser while a slow stream of oxygen was passed through the liquid. A creamish-yellow precipitate soon began to form, and after two hours the colour of the supernatant solution assumed a clear royal blue. Whereupon the mixture was poured into water (4 l.). The product was collected next day, and purified by solution in hot 2N hydrochloric acid, treatment with charcoal and reprecipitation with 2N ammonium hydroxide several times. The product formed a *pale cream solid*, m.p. > 360°. Found: C, 56·0; H, 3·2; N, 35·2; $C_{13}H_9ON_7$ requires C, 56·0; H, 3·2; N, 35·15 per cent).

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needles, m.p. 188 to 189°, from aqueous methanol. (Found: C, 58·3; H, 4·6; N, 36·8. $C_{11}H_{10}N_6$ requires C, 58·4; H, 4·5; N, 37·1 per cent).

C. 2-Amino-9(4'-chlorophenyl)8-azapurine

(i) *2-Chloro-4-(4'-chloroanilino)-5-nitropyrimidine.* 2:4-Dichloro-5-nitropyrimidine (1 0 g.) in ethanol (25 ml.) was cooled to —10° in an ice-salt bath and treated all at once with a solution of *p*-chloroaniline (0 66 g.) in ethanol (12 ml.). After a few seconds the clear solution became a mass of yellow solid, which was immediately collected and recrystallised from ethanol, to yield *2-chloro-4(4'-chloroanilino)-5-nitropyrimidine* as yellow needles, m.p. 150 to 151°. (Found: C, 42·0; H, 2·6; N, 19·65; Cl, 24·5. $C_{10}H_6O_2N_4Cl_2$ requires C, 42·1; H, 2·1; N, 19·7; Cl, 24 9 per cent).

If the reaction was attempted on a larger scale, then a mixture was obtained with the di-*p*-chloroanilino compound. For comparison an authentic sample of this was prepared from the dichloronitropyrimidine (1 g.) in ethanol (15 ml.) and *p*-chloroaniline (2·64 g.) in ethanol (50 ml.), the pyrimidine being added to the amine this time. The mixture was then boiled under reflux to complete the reaction and the product, isolated by filtration, formed pale yellow needles, m.p. 216 to 217° from *n*-butanol. (Found: C, 51·3; H, 3·0; N, 18·8; Cl, 19·3. $C_{16}H_{11}O_2N_5Cl_2$ requires C, 51·0; H, 2 9; N, 18·6; Cl, 18·9 per cent).

2-Amino-4(4'-chloroanilino)-5-nitropyrimidine. 2-Chloro-4(4'-chloroanilino)-5-nitropyrimidine (2 0 g.) was heated at 100° for three hours in a sealed tube with saturated ethanolic ammonia solution (20 ml.). The product (1·9 g.), collected on cooling, crystallised from *n*-butanol as golden-yellow leaflets, m.p. 241°. (Found: C, 45·5; H, 3·45; N, 26·3; Cl, 13 6. $C_{10}H_8O_2N_5Cl$ requires C, 45·2; H, 3·0; N, 26·4; Cl, 13·4 per cent).

2 : 5-Diamino-4(4'-chloroanilino)pyrimidine. The above nitro compound (1·32 g.) was added to a solution of stannous chloride (5·0 g.) in concentrated hydrochloric acid (25 ml.) and the mixture warmed and ground. When reduction was complete, as shown by the replacement of the orange colour by a cream suspension of the stannichloride, the product was collected, decomposed by 40 per cent sodium hydroxide and the base collected by extraction with chloroform. The residue obtained by evaporation of the extract was crystallised from hot 2N hydrochloric acid (charcoal) to yield the *dihydrochloride monohydrate* as colourless needles, m.p. 286 to 287° (decomp) (Found: C, 37·1; H, 4·5; N, 21·8; Cl, 32·7. $C_{10}H_{10}N_5Cl \cdot 2HCl \cdot H_2O$ requires C, 36·75; H, 4·3; N, 21·45; Cl, 32·65 per cent). The *monopicrate* formed lemon-yellow rosettes of prisms, m.p. 256° (decomp.) from aqueous ethanol. (Found: C, 41·8, H, 3·1; N, 23·9; Cl, 7·9. $C_{10}H_{10}N_5Cl \cdot C_6H_3O_7N_3$ requires C, 41·3; H, 2·8; N, 24·1; Cl, 7·65 per cent).

2-Amino-9(4'-chlorophenyl)-8-azapurine (CB. 2356, No. 63)

2 : 5-Diamino-4(4'-chloroanilino)pyrimidine dihydrochloride (0·4 g.) was dissolved in water (80 ml.), concentrated hydrochloric acid (2 drops)

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crystallised from 2N hydrochloric acid (charcoal) to yield the *triamine dihydrochloride* as colourless needles, m.p. 249 to 250° (decomp.). (Found: 3, 44·0; H, 4·8; N, 25·9; Cl, 25·3. $C_{10}H_{11}N_7 \cdot 2HCl$ requires C, 43·8; H, 4·75; N, 25·6; Cl, 25·9 per cent). Treatment of an ethanolic solution of the dihydrochloride with an ethanolic solution of picric acid gave the *monopicrate* as yellow needles, m p. 249 to 250° (decomp.) from aqueous ethanol. (Found: C, 44·4; H, 3·4; N, 25·6; $C_{10}H_{11}N_5 \cdot C_6H_3O_7N_3$ requires C, 44·7; H, 3·3; N, 26·1 per cent).

(iii) *2-Amino-9-phenyl-8-azapurine* (CB. 2355, No. 61). The above dihydrochloride (0 68 g.) was dissolved in water (40 ml.) containing a few drops of concentrated hydrochloric acid to suppress any hydrolysis and was stirred and cooled to 5°. A solution of sodium nitrite (0·24 g.) in water (1 ml) was added when an immediate pale yellow precipitate was formed. After half an hour, the pH of the mixture was adjusted to 8 by the addition of ammonium hydroxide and the product was collected, and crystallised from aqueous methanol (charcoal). The *triazolopyrimidine* formed colourless rosettes of needles m.p. 167 to 168°. (Found: C, 56·6; H, 3·9; N, 39·7; $C_{10}H_8N_6$ requires C, 56·6, H, 3 8; N, 39·65 per cent).

B. 2-Amino-6-methyl-9-phenyl-8-azapurine

(i) *2-Amino-4-anilino-6-methyl-5-nitropyrimidine.* 4-Anilino-2-chloro-6-methyl-5-nitropyrimidine (2·54 g.; Spickett[19], *loc cit.*) was heated at 100° in a sealed tube with saturated methanolic ammonia solution (20 ml.) for 1½ hours. When cold, the tube was opened and the product (2·3 g.) was collected and crystallised from *n*-butanol to give the *amino* compound as yellow needles, m.p. 192 to 193°. (Found: C, 53·3, H, 4·3, N, 28·6. $C_{11}H_{11}O_2N_5$ requires C, 53·9; H, 4·5, N, 28·6 per cent).

(ii) *2 : 5-Diamino-4-anilino-6-methylpyrimidine.* The nitro compound (3 0 g.) was reduced using a solution of stannous chloride (12 g.) in concentrated hydrochloric acid (60 ml.) The precipitated stannichloride was collected, decomposed with 40 per cent sodium hydroxide and the liberated base extracted with several portions of chloroform. Evaporation of the dried extracts gave the crude product as a crusty brownish solid (2·4 g.). This, on crystallisation from hot 2N hydrochloric acid (charcoal) gave colourless, lustrous needles, m.p. 239 to 240°, of the *dihydrochloride.* (Found: C, 45 8, H, 5 2, N, 24 45; Cl, 24·65. $C_{11}H_{13}N_5 \cdot 2HCl$ requires C, 45·9; H, 5·2, N, 24·3, Cl, 24·7 per cent) The *monopicrate* formed orange-yellow leaflets, m p. 252 to 253° (decomp) from dilute ethanol. (Found C, 45·9; H, 3·8; N, 24·85. $C_{11}H_{13}N_5 C_6H_3O_7N_3$ requires C, 45·95, H, 3 6; N, 25·25 per cent).

(iii) *2-Amino-6-methyl-9-phenyl-8-azapurine* (CB. 2341, No. 62). 2,5-Diamino-4-anilino-6-methyl pyrimidine dihydrochloride (2·0 g.) in water (150 ml) containing concentrated hydrochloric acid (4 drops) was cooled to 5° and treated with a solution of sodium nitrite (0·7 g) in water (2 ml) with stirring. A precipitate was formed immediately and after 15 minutes the pH was adjusted to 7–8 by the addition of ammonium hydroxide and the product collected The *azapurine* formed colourless

with 5-formyltetrahydropteroylglutamic acid (folinic acid). Stock cultures of *Str. faecalis* were maintained in the liver-tryptone agar of Nyman and Gortner[20], and stock cultures of *Leuc. citrovorum* in brewer's yeast agar. Tests were carried out in a basal medium similar to that used by Collier and Phillips[6] for testing antagonists of folinic acid, which was based on the medium of Barton-Wright, Emery and Robinson[21]. This contained: acid hydrolysed casein (A. & H.), 6 g.; L-cystine, 100 mg.; DL-tryptophane, 120 mg.; glucose, 10 g.; sodium acetate (anhydrous), 10 g.; adenine HCl, 10 mg.; guanine HCl, 10 mg.; uracil, 10 mg.; xanthine, 10 mg.; aneurine, 100 μg.; riboflavine, 200 μg; pyridoxine HCl, 100 μg.; calcium D-pantothenate, 500 μg.; D-biotin, 0·4 μg.; nicotinic acid, 100 μg.; NaCl, 5 g.; KH_2PO_4, 500 mg.; K_2HPO_4, 500 mg.; $MgSO_4·7H_2O$, 200 mg.; $MnSO_4·4H_2O$, 10 mg.; $FeCl_3$, 2 mg.; glass-distilled water, to 500 ml. The pH of this medium was adjusted to 7·0 and after steaming for 30 minutes the medium was filtered while hot.

Antifolic tests. The above medium, which was double-strength, was filled out in 5 ml. aliquots, in which solutions of test compounds were serially diluted. PGA was dissolved in a little potassium dihydrogen phosphate solution and made up to the required concentration in glass-distilled water. Five ml. quantities of PGA solution were added to aliquots of medium before autoclaving at 10 lb. for 10 min. Folinic acid was dissolved in sterile glass-distilled water and added with sterile precautions to double-strength medium after autoclaving. Inocula of bacteria were prepared from stock cultures by growing in liver tryptone broth for 20 hours at 37°. Cultures were centrifuged, washed twice with sterile saline and made up to correspond in opacity with Brown's No. 2 tube. This suspension was diluted 1:100 with saline and 0·02 ml. used as inoculum. After 60 to 64 hour incubation at 37°, growth was estimated by titration with 0·1N NaOH, using bromthymol blue as indicator. For reference, 4-amino-10-methylpteroylglutamic acid (A-methopterin) and 2.4-diamino-6:7-diisopropylpteridine (0/129) were used.

EXPERIMENTAL

Str. faecalis and pteroylglutamic acid. The technique of screening tests is illustrated in Figure 1. When *Str. faecalis* was cultivated in the presence of enough PGA to give good growth (2 ng/ml.), increasing concentrations of an inhibitor depressed growth to an increasing degree. When the level of PGA was raised to 20 ng./ml., higher concentrations of inhibitor were required to produce equal depression, indicating some degree of competitive antagonism.

The antifolic acid activities of all compounds described in the chemical section were examined by this method, using levels of 2 and 20 ng./ml. PGA. Compounds numbered 9, 12, 15, 19, 21, 22, 25, 29, 32-34, 49-58, 60-66, and 68 were inactive in saturated solution, while the remainder showed activity. The concentration of each active inhibitor required to depress growth by half at each level of PGA was determined in three or more independent experiments. The geometric means of these concentrations and their 95 per cent fiducial limits, together with the molar ratios

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was added, and the mixture cooled to 5° in ice-water. A solution of sodium nitrite (0·12 g.) in water (1 ml.) was added and the mixture was well stirred. After adjustment of the pH to 8 by means of concentrated ammonium hydroxide, the precipitate was collected and crystallised from aqueous methanol tó afford the triazolopyrimidine as rosettes of fine white needles, m.p. 236°. (Found: C, 48·7; H, 3·0; N, 34·2; Cl, 14·7. $C_{10}H_7N_6Cl$ requires C, 48·7; H, 2·8; N, 34·1; Cl, 14·4 per cent).

8-Azapurine (CB. 2354, No. 64)

4:5-Diaminopyrimidine (0·4 g.) in 95 per cent ethanol (10 ml.) was refluxed with amyl nitrite (5·5 ml., 10 mol.) for two hours, by which time the solution was nearly clear. It was cooled, filtered from a small amount of dark coloured matter which was discarded and the filtrate was evaporated to dryness to leave a brown solid. This was sublimed at 130 to 140°/1·5 mm. to yield a cream substance, smelling slightly of fatty acids. The m.p. varied with the rate of heating and standard conditions had to be adopted to obtain concordant results. The sample was placed in an electrically-heated copper block at 125° and the temperature raised at the rate of 2° per minute. The m.p. was then 170 to 171°.

The product was resublimed at 130 to 140°/1·5 mm. and the initial fraction (0·08 g.) m.p. 164 to 165° was rejected. Sublimation was continued and the second fraction (0·29 g.) had m.p. 175°. A small amount of yellowish substance was obtained subsequently and the remainder consisted of brown, involatile decomposition products. Repeated sublimation of the fraction, m.p. 175° only gave material of the same m.p. and an involatile residue. The final *sublimate* was a white solid, insoluble in ether, but very soluble in water to give a solution with a strongly acid reaction to litmus. On treatment with silver nitrate solution, this gave a heavy white precipitate of the silver salt; with sodium bicarbonate solution a vigorous effervescence is observed. (Found: C, 39·9; H, 2·6; N, 58·1; $C_4H_3N_5$ requires C, 39·7; H, 2·5; N, 57·8 per cent).

2:6-Diamino-3(4'-chlorophenylazo)pyridine (CB. 2357, No. 66)

2:6-Diaminopyridine (5·45 g.) in dilute hydrochloric acid solution (2N, 50 ml.) was cooled in ice, stirred and treated slowly with a solution of 4-chlorobenzenediazonium chloride (from *p*-chloroaniline (6·375 g.)). To the resulting dark-red solution was added crystalline sodium acetate to adjust the pH to 5–6 and the thick yellow slurry so obtained was diluted with water and filtered. The product on crystallisation from aqueous ethanol afforded the *azo compound* as yellow prisms, m.p. 184 to 185°. (Found: C, 53·45; H, 4·3; N, 27·9; Cl, 14·1. $C_{11}H_{10}N_5Cl$ requires C, 53·4; H, 4·0; N, 28·3; Cl, 14·3 per cent).

BIOLOGICAL SECTION*

METHODS

Strains. *Streptococcus faecalis* (R) was used for experiments with pteroylglutamic acid (PGA) and *Leuconostoc citrovorum* (NCTC 7837)

* By H.O.J.C. and P.L.H.

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growth-factor required for a given biological effect do not fully character-
ise competitive action. Such methods however can indicate some degree
of competition.

It will be seen from Tables III and IV that for each compound a tenfold
increase in PGA requires a twofold to eightfold increase in inhibitor to
produce the same degree of inhibition. This indicates the presence of

TABLE III

INHIBITION OF GROWTH OF *Str. faecalis* BY VARIOUS ARYL-AZOPYRIMIDINES IN THE
PRESENCE OF PTEROYLGLUTAMIC ACID (PGA). VALUES EXPRESSED AS GEOMETRIC
MEANS AND THEIR 95 PER CENT FIDUCIAL LIMITS

Compound		ug/ml to inhibit growth by half in presence of				Mean molar ratio inhibitor to PGA at		Ratio of means at 20 and 2 ng/ml PGA
No.	CB Reference No.	2 ng/ml PGA		20 ng/ml PGA		2 ng/ml.	20 ng/ml.	
		Mean	Limits	Mean	Limits			
1	2295	0·33	0·20–0 56	1 66	1 34–2·07	317 8	159 8	3·0
2	2310	0·25	0 09–0·67	1 13	0 39–3 26	209 2	94 55	4 5
3	2309	0·39	0 15–1·06	1·06	0 41–2 74	326 3	88 70	2 7
4	2377	0·21	0 08–0 59	1 08	0 45–2·61	173 7	90 37	5 1
5	2328	0·19	0 12–0 28	0 82	0 35–1·92	136 0	58 70	4 3
6	2311	0·17	0 12–0·23	0 81	0·60–1·09	121 7	57·98	4 8
7	2326	0·37	0 16–2·08	2 75	0·99–7 67	424 2	203 5	4 8
8	2307	0·41	0 14–1·17	0·71	0 50–1·00	303·4	52 53	1 7
10	2283	0·54	0 20–1·43	2 22	0·80–6 18	399 6	164 3	4 1
11	2349	0·21	0 11–0 65	0 54	0·25–1 19	153 8	39·76	2·0
13	2299	1·11	0 51–2·41	3 00	1·96–12 76	945·0	425 7	4 5
14	2323	3·67	1·30–10 35	12 88	4 60–36·03	2,964	1,040	3 5
16	2330	0·28	0 12–0 67	0 86	0·31–2 39	221 3	67 96	3 7
17	2315	0·16	0 06–0 45	0·43	0·21–0 90	129·4	33 98	2 7
18	2331	2·26	1·01–5 06	4 77	2 10–10·77	1,779	373·6	2 1
20	2298	12 28	7 25–20 81	—	—	9,568	—	—
23	2271	22·41	10 95–43 64	7 60	28·45–49 68	13,650	2,290	1·7
24	2266	7 72	3 08–19 36	—*	—	5,840	—	—
26	2260	14 59	7 02–30 34	22·40	12 66–39 64	9,153	1,405	1·6
27	2280	0·49	0 18–0 64	1 10	0·46–2 63	408 5	91 70	2 3
28	2316	0·76	0 53–1·09	1·65	1·15–2 36	542 3	137 7	2 2
30	2329	0·13	0 05–0 39	0 54	0·27–1·09	115 3	47·90	4 2
31	2304	20·72	18 02–24 39	52·94	27 30–102·0	18,310	4,879	2 6

* Incomplete or no inhibition in saturated solution.

TABLE IV

INHIBITION OF GROWTH OF *Str. faecalis* BY VARIOUS 8-AZAPURINES AND OTHER
COMPOUNDS IN THE PRESENCE OF PTEROYLGLUTAMIC ACID (PGA) VALUES EXPRESSED
AS GEOMETRIC MEANS AND THEIR 95 PER CENT FIDUCIAL LIMITS

Compound		ug/ml. to inhibit growth by half in presence of				Mean molar ratio inhibitor to PGA at		Ratio of means at 20 and 2 ng/ml PGA
No	CB Reference No	2 ng/ml. PGA		20 ng/ml. PGA		2 ng/ml.	20 ng/ml.	
		Mean	Limits	Mean	Limits			
35	2314	1 16	0 62–2 15	5 43	4 24–6·93	1,126	527 5	4 7
36	2321	0·35	0 13–0·93	1·96	1 66–2 32	295 1	165 3	5 6
37	2308	0·62	0 26–1 52	4·68	1·75–12 51	522 9	394 6	7 6
38	2278	0·12	0 07–0·21	0·85	0 36–2·01	101 2	71 67	7 1
39	2333	0·46	0 26–0 84	1 33	1 53–2 18	331 2	131·9	4 0
40	2319	0·12	0 05–0 30	0·59	0·28–1 24	86·47	42 52	4 9
41	2333	0·22	0 09–0 58	0·73	0 34–1 58	163·9	54 38	3 3
42	2318	0·12	0 05–0·29	0·57	0·24–1 37	83 39	42 46	4 8
43	2324	0·19	0 12–0·28	1 20	0·44–3 22	141 5	89 39	6 3
44	2282	0·22	0 08–0 63	1·42	0·53–3 78	163 9	105 8	6 5
45	2364	0·04	0 02–0·10	0·11	0 04–0 30	22·90	6 30	2 8
46	2350	0·51	0 41–0 64	1·64	0·58–4 65	242·4	77 93	3 2
47	2327	0·98	0 37–2 63	2 71	0·98–7·45	797·4	220 5	2 8
48	2335	0·04	0 03–0·07	0·19	0·09–0 38	31 84	15 12	4 8
59	2381	0·10	0 04–0·24	0·42	0 22–0 81	79 32	33 32	4 2
67	2316	0·57	0 24–1 36	3·89	2·67–5 66	897 8	612 7	6 7
A-Methopterin		0·0005	0·0002–0·0012	0·0023	0·0013–0·0041	0·0226	0 112	4 6
Pteridine 0/129		0·0026	0·0011–0·0060	0·0143	0·0071–0·0290	2 301	1 286	5 5

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of inhibitor to PGA based on these mean values, and the ratios of the mean inhibitory concentrations at 20 and 2 ng./ml. PGA are expressed for aryl-azopyrimidines in Table III. Corresponding results for 8-azapurines, Compound 67, A-methopterin and pteridine 0/129 are given in Table IV. From these Tables it will be seen that the most active compounds among azopyrimidines were Nos. 5, 6, 11, 17 and 30 and among azapurines the considerably more potent Nos. 45 and 48. Compound 45 had the highest potency, which was about one-fiftieth that of A-methopterin in the same test.

Leuc. citrovorum *and folinic acid.* Replacing *Str. faecalis* by *Leuc. citrovorum,* some of the compounds antagonising PGA were screened at levels of 0·92, 4·6 or 23 ng./ml. folinic acid. Of the aryl-azopyrimidines tested, only compounds 1, 13 and 26 showed some degree of *competitive anta*gonism, the remainder tested (Nos. 2, 3, 4, 6, 8, 10, 14, 16, 17, 20, 23, 24 and 27) being inactive. All the azapurines tested (Nos. 35, 37, 38, 40, 42 and 48) were inactive and so was Compound 67. A-methopterin had about one-hundredth and Compound 1 about one-fifth of their respective potencies with *Str. faecalis.*
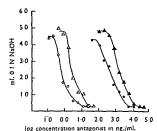
FIG 1   Growth inhibition of *Str. faecalis* in the presence of pteroylglutamic acid (PGA)
●   Compound 1 at 2 ng /ml. PGA
▲   ,,   ,, 20   ,,   ,,
○   A-methopterin at 2 ng./ml PGA
△   ,,   ,, 20   ,,   ,,

DISCUSSION

In 1914 Langley[22] showed that for any particular muscle of the frog, the ratio of curare (antagonist) to nicotine (agonist) necessary to prevent a response was constant and independent of the levels of concentration. In 1940, a similar constancy of ratio between sulphanilamide and aminobenzoic acid for *Str. pyogenes* was described by Woods[23], who ascribed it to competition between those substances for an enzyme. Since then, constancy of ratio between antagonist and agonist has commonly been used as a test of competition, particularly in bacteria. Schild[24], however has argued that "the mass action equation as developed by Gaddum[25] for a first order reaction requires a ninefold increase of antagonist corresponding to a five-fold increase of active drug. . . . Straight proportionality between drug and antagonist at low concentrations of the antagonist is presumptive evidence against the existence of a simple mass action relation" The above divergent viewpoints and the work of Timms[26] suggest that methods based simply on measuring ratios of antagonist to

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structure this substituent might be concerned in facilitating attachment to an enzyme. Modification of the two active structures by substitution of a pyrimidine by a pyridine ring as in VIII (No. 60) and IX (No. 66) led to inactivity.

In the aryl-azopyrimidines (III) activity is consistent with either one, two or three amino substituents in the pyrimidine ring, but for maximal effect three amino groups are generally desirable. The effects of substitution in the benzene ring with chlorine or bromine depend on the position and number of the substituent atoms. A single atom in the *para* position was beneficial, but dihalogen compounds were usually less active than the mono-substituted and the tribromo compound (No. 12) was inactive.

Of the twenty-five 8-aryl-8-azapurines tested, fifteen contained two amino groups in the pyrimidine ring and all were active. The remaining ten compounds contained one or no amino groups. Since none of these was active, we conclude that two amino groups are essential for activity in this series. In the azapurines, the effects of substitution in the benzene ring with chlorine or bromine differed in some ways from those in the azopyrimidine series. Substitution of a single halogen atom in any position increased activity, and the dihalogen compounds were generally more active than the monosubstituted. On the other hand, as in the azopyrimidine series, the *para* position in the monosubstituted derivatives was the most beneficial, and the tribromo derivative (No. 46) was much less active than the dibromo compound (No. 45).

The active compounds of the 8-azapurine series, some of which are more potent than any of the azopyrimidines tested, provide what appear to be the first examples of antifolic acid activity among 8-azapurines.

SUMMARY

1. Thirty-four amino-5-aryl-azopyrimidines and thirty amino-8-aryl-8-azapurines and a few examples of three related structures were synthesised as potential antifolic acids because of a structural relation to pteridine.

2. Screened with *Str. faecalis* and pteroylglutamic acid (PGA), 23 arylazopyrimidines and fifteen 8-azapurines showed antifolic acid activity. This was greatest in Compound 45, which possessed about one-fiftieth the potency of A-methopterin in the same test.

3. With *Leuc. citrovorum* and folinic acid, 3 of 16 azopyrimidines and none of 6 azapurines tested showed antifolic acid activity. A-methopterin had about one-hundredth and Compound I about one-fifth of their respective potencies with *Str. faecalis*.

Consideration of the activities of the above compounds has enabled certain structure-activity relations to be deduced.

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some degree of competitive antagonism of pteroylglutamic acid. A full test of competition depends on analysis of curves of log concentration of folic acid plotted against bacterial growth in the absence of and in the presence of various levels of inhibitor. Such curves have shown that Compound 1 competes with both PGA and folinic acid. These curves and others at present being obtained for further compounds will be published elsewhere.

<div align="center">STRUCTURE-ACTIVITY RELATIONS</div>

The fact that the 4-amino-5-aryl-azopyrimidines (III) can be readily oxidised, by chemical agents, to the 8-aryl-8-azapurines (IV) and that both series of compounds contain active substances suggested that the activity of the azo compounds might be due to this conversion by oxidation during bacterial metabolism. Detailed investigation however revealed a marked lack of the relation which, on this hypothesis, would have been expected between the activities of corresponding compounds in the two series. For example, for *Str. faecalis* and PGA, certain 8-azapurines (Nos. 49, 50 and 55) which are substituted with amino and hydroxy groups at the 2 and 4 positions (IV, R″ = NH_2, R′ = OH) respectively, are inactive, whilst the corresponding azo compounds (Nos. 27, 28 and 31) are active. Again for *Leuc. citrovorum* and folinic acid the 8-azapurine No. 35 is inactive, but the corresponding azopyrimidine (No. 1) is active. On the other hand, for *Str. faecalis*, certain highly active 8-azapurines, e.g., Nos. 43 and 46, correspond to the inactive azopyrimidines, Nos. 9 and 12 respectively. A further discrepancy is revealed by comparison of the activities of three azopyrimidines (Nos. 2, 3 and 4), which differ only in the position of the chlorine atom in the benzene ring, and the corresponding 8-azapurines (Nos. 36, 37 and 38). The azo compounds are of comparable activity whilst the 8-azapurines showed more fluctuation.

An alternative hypothesis for the activity of the azopyrimidines was that they might be reductively split (a process known to occur *in vivo* with a variety of azo compounds[27-30]) to yield a 4:5-diaminopyrimidine (V) which might be the active form; but, although 2:4:5:6-tetraminopyrimidine (V, R = NH_2, No. 67), which might be derivable from many of the active compounds, was an antifolic acid, the inactivity of 2:4:5-triamino-6-hydroxypyrimidine (V, R = OH, No. 68), also derivable from the ... ... l and 31, rendered the hypothesis untenable. ... ... the antifolic activity found in the azopyrimidines and 8-azapurines is intrinsic for each series.

Simplification of the diamino-8-aryl-8-azapurines by removing the aryl substituent to produce 2:6-diamino-8-azapurine (No. 65) or by further removing the amino groups to give 8-azapurine itself (VI, No. 64) led to inactive compounds. The inactivity of No. 65 thus suggests that an aryl substituent may be necessary. Since, also, the three 9-aryl-8-azapurines prepared (VII, R′ = H, R″ = C_6H_5, No. 61; R′ = CH_3, R″ = C_6H_5, No. 62; R′ = H, R″ = C_6H_4 Cl *p*, No. 63) were all inactive, it may be that the particular position of the aryl substituent in the 8-aryl-8-azapurine structure relative to the pyrimidine ring is also important. As a flat

ANTIFOLIC ACIDS AND ANTIPURINES IN CHEMOTHERAPY

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IN this review an attempt will be made to illustrate the uses and potential uses of antifolic acids and antipurines in chemotherapy. These two types of agent can probably be best defined, in brief, by referring to their ability to inhibit the growth of various organisms; this inhibition can be prevented or reversed by the addition respectively of folic acid (I) or one of the natural purines, usually adenine (II; $R' = NH_2$, $R'' = H$) or guanine (II; $R' = OH$, $R'' = NH_2$). Antagonism experiments in small animals have proved difficult to assess accurately, and the most commonly employed organisms are bacteria, e.g., *Lactobacillus casei*, *Streptococcus faecalis* and *Escherichia coli*. By their use we can ascertain whether the ratio of the potential antagonist to the amount of folic acid, or the purine, required to relieve inhibition remains roughly the same whatever the actual concentrations of the antagonistic substances. In this instance a true competitive antagonism would exist and, when present, constitutes usually the best available evidence for a true folic acid or purine antagonist, since when the antagonism is irreversible or not competitive, we do not know whether the metabolite is essentially involved or not, although the bacteria concerned may normally require folic acid or a purine for growth. It must be emphasised that these simple and ideal conditions seldom, if ever, apply wholly in practice, and they are here used mainly as an illustration of the principle of competitive antagonism and the logical criterion for its presence. For an example of the detailed treatment of this subject the reader may be referred to reference 1. In this review, except where stated, only reversible antagonisms will be considered.

FOLIC ACID ANTAGONISTS
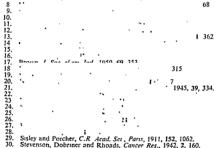
Antifolic acids and antipurines are logically related, since recent work on the biosynthesis of nucleic acids[2–4] reveals the essential role of folinic acid (Citrovorum factor) in the synthesis of their purine constituents. This derivative of folic acid is necessary for the synthesis of glycinamide ribotide, and at a later stage the ring closure of 5-amino-4-iminazole-carbonamide ribotide. Folinic acid (5-formyl-5:6.7:8-tetrahydropteroyl-glutamic acid, III) is formed from folic acid, and it has been shown that, for example, the two powerful antifolic acids, aminopterin (IV; $R' = NH_2$, $R'' = H$) and amethopterin (Methotrexate IV; $R' = NH_2$, $R'' = Me$) inhibit growth by preventing the conversion of folic to folinic acid in various organisms. In these cases also there is probably a direct interference with folinic acid[5–8].

The first chemotherapeutic developments in this field were the successful use of aminopterin and Methotrexate in inducing in some proportion,

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TWO NEW SERIES OF ANTIFOLIC ACIDS

REFERENCES

1. Burchenal, Robinson, Johnston and Kushida, *Science*, 1950, 111, 116.
2.
3.   *em.,* 1947, 169, 689.
4.
5.   *nd ,* 1950, 165, 1004.
6.
7.   V⁙ ⁙h Congrès Inter-
8   68
9.
10.
11.
12.
13.   I 362.
14
15.
16.
17. Brown *J Soc ol am Ind* 1950 69 353
18   315
19.
20.   I ⁙⁙ 7
21.   ⁙ 1945, 39, 334.
22.
23
24.
25.
26.
27.
28.
29. Sisley and Porcher, *C.R Acad. Sci , Paris*, 1911, 152, 1062.
30. Stevenson, Dobriner and Rhoads, *Cancer Res.*, 1942, 2, 160.

(I)

(II)

(III)

(IV)

(V)

(VI)

(VII)

(VIII)

(IX)

(X)

(XI)

(XII)

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remissions in children with acute leukaemia[10]. Rather less useful are the folic acid analogues which contain a hydroxy group in the 4-position, e.g. (IV; R' = OH, R'' = Me) and they have now fallen out of general clinical use. In general, the clinical effect of these antifolic acids is reflected in experiments with leukaemia in mice[11-15]. The fact that the clinical use of antifolic acids does sooner or later induce a drug resistance is a serious disadvantage. This effect has been alleviated in experimental leukaemias by the combination of two drugs which do not show cross-resistance, e.g., by the use of 6-mercaptopurine (*vide infra*) with Methotrexate[16]. Similar clinical attempts have so far been promising.

The field of the antifolic acids may still, nevertheless, be worth cultivating further, since Farber[17] has found that drugs of the Methotrexate type have produced impressive improvement in rhabdomyosarcoma, Hodgkin's disease, lymphosarcoma, neuroblastoma and chronic lymphoid leukaemia. Also, Colsky[18] reported improvement in carcinoma of the breast treated with Methotrexate.

Amongst simpler types of folic acid analogues which have yielded, at the least, significant antifolic activity, are the 6:7-substituted diaminopteridines (V). In this type R' and R'' have been phenyl or substituted phenyl[19,20], R' phenyl or substituted phenyl and R'' amino[21] or R' and R'' have both been alkyl or aralkyl, or R' aryl and R'' alkyl[22-24]. Antifolic acid activity has also been found in more complex types, for example, the naphthopteridine (VI)[25,26] and the indolopteridine (VII; R=alkyl)[22-24]. None of these types has yet yielded compounds of value in leukaemia, but high antimalarial activity has been found in some of the 2:4-diamino-6:7-dialkylpteridines and 2:4-diamino-6-aryl-7-alkylpteridines; e.g., V (R' = anisyl, R'' = *iso*propyl) which equals proguanil (Paludrine) in activity. But for the emergence of the pyrimidine derivative pyrimethamine (Daraprim, *vide infra*), a drug of wide usefulness clinically would probably have arisen from this series.

Another potentially interesting type of activity was found both in the 6:7-dialkyl-2:4-diaminopteridines and in the indolopteridines, (e.g., VII; R = Me) where substances with high activity *in vitro* against *Vibrio cholerae* were found.

High antifolic and antimalarial activity has also been found in structures simpler than pteridines, viz., the 2·4-diamino-5-chlorophenyl pyrimidines[27] (e.g., VIII), and the 4·6-diamino-1-chlorophenyl-1·2-dihydrotriazines (e.g., IX)[28-32]. 2:4-Diamino-5-3':4'-dichlorophenyl-6-methylpyrimidine (VIII; R = Me) resembles aminopterin and Methotrexate in its antifolic acid properties, but it has had only slight success in the treatment of acute leukaemia in children[33] and seems to be inferior to Methotrexate owing to its toxicity. Slight inhibition of growth of the mouse sarcoma 180 has been caused by this type of compound where the methyl group is replaced by hydrogen, ethyl or *n*-amyl groups[34]. Of much greater clinical interest in this group is pyrimethamine[35] (Daraprim, VIII; R = C_2H_5), a widely used antimalarial drug.

The series of dihydrotriazines (e.g., IX), studied by Modest and Foley and their collaborators, are non-competitive inhibitors of folic acid and

The most complex antipurine of any so far produced is puromycin, 6-dimethylamino-9-(3'-*p*-methoxy-L-phenylalanyl-amino-3'-deoxy-D-ribosyl)purine (XV). This evinces an antiguanine action in *E. coli*[33] and it has been shown that its trypanocidal action in *T. equiperdum* was due to interference with the purine metabolism of the parasite[49]. This trypanocidal action was reversed by adenine and other purines[50]. After showing activity against experimental tumours and leukaemias, all clinical trials in various malignant diseases were disappointing; but effectiveness as an amoebicide was uncovered first in guinea pigs[52] and then in man[53]. When the structure of puromycin was simplified by removal of the *p*-methoxyphenylalanyl residue (at the dotted line shown in XV) the amine obtained, 6-dimethylamino-9-(3'-amino-3'-deoxy-D-ribosyl) purine was similar to puromycin in its activity against experimental tumours and trypanosomiasis. Like puromycin, this compound has not as far shown promise in clinical trials with malignant disease.

New types of purines and azapurines have recently been synthesised as potential antipurines[54] on the hypothesis that cyclic substituents in the 9 position might be of value since they bear a spacial relation to the furanose ring (cf. XVI) which appears, in the form of the phosphate ester, as a stage in the normal biosynthesis of the hypoxanthine and presumably other purine moieties in ribonucleic acid, and possibly as the deoxy form in deoxyribonucleic acid.

The formulae XVII to XIX illustrate four active compounds, all of them showing essentially an antiguanine type of activity when tested with *E. coli*[55]. The azapurine (XVII) is about three times as active in this respect as is 6-mercaptopurine. The other compounds are less active; XVIII (R = Me or H) also show a less competitive type of action than does XVII, and XIX has a less competitive kind of inhibition than XVIII.

Of purine analogues, a little more removed from purine in structure, a series of thiazolopyrimidines (3:4:6-triaza-1-thiaindenes) (e.g., XX) and a series of pyrazolopyrimidines (1:2:5:7-tetrazaindenes) (XXI) have yielded active compounds. In the 1:2:5:7-tetrazaindene series[57] activity against those experimental tumours usually susceptible to the action of antipurines has been found[58]. In *E. coli*, XXI (R = Me) is about three times weaker than 6-mercaptopurine as an antiguanine, and had very little adenine activity[59]. Very recently, partially competitive antiguanine activity has been found in the 8-thiapurine derivative XXII[60]. Also, in the 2-azapurine series, 2-azaadenine (XXIII; R = NH₂) and 2-azahypoxanthine (XXIII; R = OH) are antipurines[62].

Benziminazole (XXIV) and its derivatives and benzthiazole (XXV) and benztriazole (XXVI) are the antipurines furthest removed from a structural analogy with purine. Benzimimazole was first found to have anti-adenine activity in various organisms[53], and an antiguanine activity in *E. coli* was found later[55]. Benzthiazole and benztriazole were similarly found to be weak antiguanines[55]. Comparison of benztriazole with 8-azapurine (XXVII)[1] showed that the latter had a similar weak, mainly antiguanine, type of activity. Thus, in this instance, substitution of the

folinic acid. Although active against experimental leukaemias, clinical experiments with these compounds have been found unpromising possibly because an adequate dose cannot be tolerated[36]. The series has provided many compounds active against experimental malaria[37,38] and experimental tumours[39], but although compound IX is the active metabolite[40] of the widely used antimalarial proguanil (X), and has been found to be more active in experimental animals, its clinical use is at least uncommon, and the same applies to the other members of the series.

Recently discovered antifolic acids are the amino-5-arylazopyrimidines (XI) and the amino-8-aryl-8-azapurines (XII)[1,41]. In neither series is activity high. Of the arylazopyrimidines the 2'.4'-dibromophenyl- and the β-naphthyl-analogues were the most active, having about 1/300th the activity of Methotrexate. The most active of the 8-azapurines was the 2':4'-dibromophenyl-analogue which had 1/60th of the activity of Methotrexate, tested with *Str. faecalis*. However, antifolic acid activity associated with the 8-azapurine structure is apparently a new observation. The possibility that folic acid fulfils an essential role in the multiplication of viruses has not been neglected. Psittacosis virus (6BC) in chick embryonic tissue was inhibited by aminopterin, Methotrexate and amino-an-fol, which is an analogue of aminopterin (IV; R' = NH₂, R" = H) differing only by the substitution of an aspartic acid for the glutamic acid moiety. Since the inhibition caused by the last two compounds was annulled by Citrovorum factor, there is evidence that the effect arises from a direct interference with the folic acid metabolism of the virus[41a].

ANTIPURINES

In the field of antipurines the number of compounds and types of structure discovered with appreciable activity has been much smaller than amongst the antifolic acids. The first example to show any marked activity as an antagonist of adenine in *L. casei* was 2.6-diaminopurine (XIII; R' = R" = NH₂)[41b]. The clinical trials[42] in leukaemia were disappointing, although a significant effect had been observed on a transplanted mouse leukaemia.[43]

At about this time the antiguanine activity of 8-azaguanine (XIV; R' = OH, R" = NH₂) was discovered with *Tetrahymena* as the organism,[44] but clinically its effect has been disappointing in trials spread over some six years, and its attempted use seems to have been abandoned.

Of the simple purines, 6-mercaptopurine[45] (XIII; R' = SH, R" = H) is by far the most interesting since it is the drug of choice in the treatment of acute leukaemia in adults[46] Also it is effective in children and in those instances in which resistance has been induced to the antifolic acids and to cortisone. It is also of some value in chronic myeloid leukaemia. 6-Chloropurine (XIII; R' = Cl, R" = H) has been tried clinically in chronic and acute leukaemia without marked success[47]. Similarly, trials of 2-amino-6-mercaptopurine (XIII, R'=SH, R"=NH₂)[47] and purine itself (XIII, R' = R" = H), which is an adenine antagonist, have revealed, at most, no advantage over 6-mercaptopurine[18].

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benzene ring by the pyrimidine ring, which is chemically very different and resembles much more a 2:4-dinitrobenzene ring, made little difference.

Antipurines and Viruses

It is convenient at this point to consider the effect of antipurines on viruses. 8-Azaguanine (XIV; R' = OH, R'' = NH₂) has been extensively investigated and causes a marked inhibition of systemic development of lucerne mosaic virus in *Nicotiana glutinosa*, and tobacco and cucumber mosaic virus in cucumbers and *Nicotiana sp.*[64]. 8-Azaadenine (XIV; R' = NH₂, R'' = H) has also some inhibitory effect on lucerne mosaic, cucumber mosaic and tobacco mosaic viruses. The virus inhibition by 8-azaguanine can be reversed by spraying the plants with adenine, guanine or hypoxanthine indicating an antipurine mechanism for the inhibition. Furthermore, 8-azaguanine is incorporated into the virus nucleic acid[65] and the infectivity of the virus was markedly reduced[65].

Vaccinia virus in tissue culture was also inhibited by 2:6-dichloro-8-hydroxypurine (XXVIII; R = R' = Cl, R'' = H, R''' = OH), 2:6:8-trichloropurine (XXVIII; R = R' = R'' = Cl, R'' = H) and 2:6-dichloro-7-methylpurine (XXVIII; R = R' = Cl, R'' = Me, R''' = H), but the inhibition was not reversed by normal purines[66].

2:6-Diaminopurine (XXVIII; R = R' = NH₂, R'' = R''' = H) was more active and its action was reversed by adenine and hypoxanthine[66]. Benzimidazole[67-70] and derivatives of benzimidazole have, however, led to more interesting results than purine derivatives or close analogues, and a detailed and systematic exploration of structure-activity relations in this field has been carried out[71,72]. By the attachment of a ribose moiety to the most active of the methyl and chlorine substituted benzimidazoles, even more active compounds were obtained of which 4:5:6-trichloro-1-β-D-ribofuranosylbenzimidazole (XXIX) was the best, being 760 times more active than benzimidazole in the inhibition of Lee

(XXVI)   (XXVII)   (XXVIII)

(XXIX)   (XXX)   (XXXI)   (XXXII)

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(XIII)

(XIV)

(XV)

(XVI)

(XVII)

(XVIII)

(XIX)

(XX)

(XXI)

(XXII)

(XXIII)

(XXIV)

(XXV)

inhibitor which produced inhibition, no effect on nucleic acid biosynthesis could be detected. Mechanisms other than an effect on nucleic acid must therefore be sought[79] and the inhibitors may well interfere in some way with co-enzyme A[80,81], pantothenic acid[81] or with diphosphopyridine nucleotide (DPN)[82]. In this connection it should be noted that co-enzyme A contains an adenine moiety linked via ribose and phosphoric acid to the pantothenic acid amide of β-mercaptoethylamine, whilst DPN also contains an adenine residue linked through ribose and phosphate to nicotinamide.

Hitherto there has been a tendency, when attempting to design new drugs intended to improve on the results so far obtained with antipurines, to consider only the structures of the established links in the chain of nucleic acid synthesis, or smaller moieties, e.g., purines, derivable therefrom, and then to think of analogous structures which might interfere with their function. A probably important consequence of the recent evidence on the mechanism of the inhibitory action of 8-azaguanine and 6-mercaptopurine will be to focus more attention on the possibility of interfering with the action of the two co-factors mentioned above and also with others.

The relation of the action of the sex hormones to the metabolism of folic acid and purines has received very little attention except from the work of Hertz and Tullner[84]. They found that the growth of chick oviduct, a tissue dependent on oestrogens, is much stimulated by administration of stilboestrol and folic acid and that this effect is competitively reversed by aminopterin, Methotrexate and 4-aminopteroylaspartic acid (amino-an-fol.). Similar results were obtained with the uterus of the sexually immature rat. Similarly an antagonism between an antipurine (2:6-diaminopurine) and adenine was demonstrated. Analogous antagonisms were observed between certain steroid hormones[81,85], and the results of this and further work might well help to explain, for example, why both antifolic acids and cortisone are beneficial in acute leukaemia.

SUMMARY

A review of the field of antifolic acids and antipurines made with the intention of illustrating their use, or potential use, in chemotherapy, reveals drugs useful, but by no means ideal, for the palliative treatment of leukaemia, and the discovery of one valuable antimalarial drug, which is an antifolic acid. There is also evidence that an antipurine mechanism can be involved in trypanocidal and also in antiviral action, but successful clinical applications of these leads have not yet been achieved. Again, there is a preliminary indication that antifolic acids might perhaps be of some value as antiviral agents and here, as with the antipurines, further work will doubtless be stimulated by the enormous importance of a successful chemotherapy of virus diseases, which is yet to be achieved.

REFERENCES

1. Timmis, Felton, Collier and Huskinson, *J. Pharm Pharmacol*, 1957, **9**, 46.
2. Schulman and Buchanan, *J. biol Chem.*, 1952, **196**, 513.
3. Greenberg, *Fed. Proc*, 1953, **12**, 651.

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influenza virus in chorio-allantoic membrane tissue culture[73,74]. Although no unequivocal evidence was obtained that the mechanism of action involved an antipurine or antinucleoside effect, it was thought, after elimination of other possible mechanisms, that the action is intracellular and involves disturbance of the metabolism of ribosides. It must be added that no purine analogue or benziminazole derivative has so far been successfully used clinically in a virus disease.

Mechanism of Action of Antipurines

A great deal of work has been directed towards elucidating the essential mechanism of action by which the antipurines inhibit growth. This may be conveniently divided into the conception of the antipurine being incorporated into the nucleic acid molecule, thus producing dysfunction, and the concept that the agent interferes initially with some enzyme or co-enzyme concerned in some way with growth or interferes with the synthesis of nucleic acid from preformed purines. The evidence suggests that any of these mechanisms can operate according to the particular antipurine and also to the organism involved.

8-Azaguanine, and also 8-azaxanthine, are incorporated principally into the ribonucleic acid (RNA) of *B. cereus*, the azaxanthine being converted into azaguanine[75]. Incorporation into the deoxynucleic acid (DNA) was only slight. In both instances marked inhibition of growth occurred. Furthermore, comparison of the effects of 8-azaguanine, 8-azaadenine, 8-azahypoxanthine, 8-azaisoguanine (XXX), 4-aminotriazole-5-carbonamide (XXXI; R = NH$_2$), 4-aminotriazole-5-carboxylic acid (XXXI; R = OH) and 5-hydroxytriazole (XXXII) on tobacco mosaic virus and *E. coli* showed that where growth inhibition occurred, so did incorporation into RNA and where there was no inhibition RNA was not incorporated[76]. Since the evidence is strong that RNA plays, at least, a much bigger part in protein synthesis than does DNA, the comparison of the actions of these closely related compounds provides good evidence for associating incorporation with growth inhibition. Again with 8-azaguanine in *E. coli*, inhibition of growth does not begin until one to two generation times after the inhibitor has been added. The time lag in the onset of growth inhibition, which does not occur with other bacterial growth inhibitors, suggested that time was necessary for the accumulation of damaged and ineffective RNA before an effect on growth was evident. On the other hand, a comparison with the inhibition of growth of *B. cereus* by azaguanine shows that for a roughly equivalent amount of inhibition much less azaguanine is incorporated into the RNA[77]. This result suggests that another mechanism may be playing a larger part in inhibiting *B. cereus* than is seen with *E. coli*. This idea has been confirmed by experiments with 8-azaguanine (labelled with ^{14}C at the carbon in the 4-position) and *B. cereus*, from which it was concluded that inhibition was produced either by the incorporation of the inhibitor into a particularly sensitive portion of the nucleic acid or by interference with some co-factor[78]. Study of the effect of 6-mercaptopurine on the growth of *E. coli* showed that at low concentrations of the

55. Collier and Huskinson, *The Chemistry and Biology of Purines*, J. and A. Churchill, London, 1957, p. 134.
56. Hitchings, Elion and Sherwood, *Fed Proc*, 1950, 9, 185.
57. Robins, *J. Amer. chem. Soc.*, 1956, 78, 784
58. Skipper, Robins and Thomson, *Proc. Soc. exp. Biol.*, *N.Y.*, 1955, 89, 594.
59. Collier and Huskinson, 1956, unpublished.
60. Timmis, *J. chem. Soc*, 1957, in the press.
61. Woolley and Shaw, *J. biol. Chem.*, 1951, 189, 401.
62. Shaw and Woolley, *ibid.*, 1952, 194, 640.
63. Woolley, *ibid*, 1944, 152, 225.
64. Matthews, *J. gen. Microbiol.*, 1953, 8, 277.
65. Matthews, *ibid*, 1954, 10, 521.
66. Thompson, Price, Minton, Elion and Hitchings, *J. Immunol*, 1950, 65, 529.
67. Brown, *ibid.*, 1952, 69, 441.
68. Thompson, *J. Bact.*, 1946, 51, 629.
69. Thompson, *J. Immunol.*, 1947, 55, 345.
70. Morgan, *J. exp. Med.*, 1952, 95, 277.
71. Tamm, Folkers, Shunk, Heyl and Horsfall, *ibid.*, 1953, 98, 245.
72. Tamm, Folkers, Shunk and Horsfall, *ibid*, 1954, 99, 227.
73. Tamm, *Science*, 1954, 120, 847.
74. Tamm, Folkers and Shunk, *J. Immunol.*, 1956, 72, 54.
75. Mathews and Smith, *Nature, Lond*, 1956, 177, 271.
76. Mathews and Smith, *Biochem. J.*, 1954, 57, vi.
77. Mathews, *Chemistry and Biology of Purines*, J. and A. Churchill, London, 1957, p 270.
78. Mandel, *J. biol. Chem.*, 1957, in the press.
79. Bolton and Mandel, *J. Pharmacol.*, 1957, in the press.
80. Biesele, *Ann N.Y. Acad. Sci.*, 1954, 60, 228.
81. Garattini, Mopurgo and Passerini, *Giorn. ital. Chemterap*, 1955, 2, 29.
82. Cahari and Garattini, *ibid.*, 1955, 2, 23.
83. Kaplan, Goldin, Humphreys, Ciotti and Stolzenbach, *J. biol. Chem*, 1956, 219, 287.
84. Hertz and Tullner, *Ann. N.Y. Acad. Sci*, 1950, 52, Art. 8, 1260.
85. Hertz, *Antimetabolites and Cancer*, American Association for the Advancement of Science, Washington, 1955, p. 253.

4. Hartman, Levenberg and Buchanan, *J. Amer. chem. Soc.*, 1955, 77, 501.
5. Nichol and Welch, *Proc. Soc. exp. Biol., N.Y.*, 1950, 74, 52, 403.
6. Sauberlich, *J. biol. Chem.*, 1949, 181, 467.
7. Hendlin, Koditschek and Soars, *J. Bact.*, 1953, 65, 466.
8. Broquist, Kohler, Hutchison and Burchenal, *J. biol. Chem.*, 1953, 202, 59.
9. ‘   •   Proc *Soc. exp. Biol., N.Y.*, 1953, 83, 272.
10.
11.   *Soc. exp. Biol., N.Y.*, 1949, 71, 107.
12.   *Meyer, Cancer Res.*, 1950, 10, 762.
13. Law, Dunn, Boyle and Miller, *J Nat. Cancer Inst.*, 1949, 10, 179.
14. B
15. B
16. S·
17. F
18. Colsky, *Chemistry and Biology of Pteridines*, J. and A. Churchill, London, 1954, p. 413.
19. Daniel, Norris, Scott and Heuser, *J. biol. Chem.*, 1947, 169, 689.
20. Daniel and Norris, *ibid.*, 1947, 170, 747.
21. Spickett and Timmis, *J. chem Soc.*, 1954, 2887
22. Campbell, Dunsmuir and Fitzgerald, *ibid.*, 1950, 2743.
23. Campbell, Collier, Dunsmuir and Fitzgerald, British Patent No. 666,788/1952.
24. Dunsmuir, British Patent Application No. 6514/1954.
25. Felton and Timmis, *J. chem. Soc*, 1954, 2881.
26. Collier, 1954, unpublished.
27. Hitchings, Falco, Vanderwerff, Russell and Elion, *J biol. Chem*, 1952, 199, 43
28. Modest, Foley, Pechet and Farber, *J Amer. chem. Soc.*, 1952, 74, 855.
29. Modest, Farber and Foley, *Proc. Amer. Assoc. Cancer Res.*, 1954, 1, 33.
30. Modest, *J org. Chem*, 1956, 21, 1.
31. Foley, *Proc. Soc. exp. Biol., N.Y.*, 1953, 83, 733, 740
32. Carrington, Crowther and Stacey, *J. chem Soc.*, 1954, 1017.
33. Murphy, Ellison, Karnofsky and Burchenal, *J. clin Invest.*, 1954, 33, 1388
34. Clarke, Buckley, Sternberg, Stock, Rhoads and Hitchings, *Cancer Res*, 1952, 12, 255.
35. Falco, Goodwin, Hitchings, Rollo and Russell, *Brit J. Pharmacol*, 1951, 6, 185
36. Farber, Toch, Sears and Pinkel, *Advances in Cancer Research*, Academic Press, New York, 1956, 4, p. 40.
37. Hewitt, Wallace, Gumble, White and Williams. *Amer. J trop Med. and Hyg.*, 1954, 3, 225.
38. Schmidt, Loo, Fradkin and Hughes, *Proc Soc. exp. Biol., N Y*, 1952, 80, 367.
39. Farber, Appleton, Downing, Heald, King and Toch, *Cancer*, 1953, 6, 135.
40 Cafrington, Crowther, Davey, Levi and Rose, *Nature, Lond.*, 1951, 168, 1080
41. Modest, Schlein and Foley, *J Pharm Pharmacol*, 1957, 9, 68.
41a. Morgan, *J exp Med.*, 1952, 95, 269.
41b. Hitchings, Elion, Vanderwerff and Falco, *J biol. Chem*, 1948, 174, 765
42 Burchenal, Johnston, Kushida, Latham, Myers, Escher, Craven, Dargeon, Southam, Moore, Hewlett, Tan and Karnofsky, *Cancer*, 1949, 9.
43. Burchenal, Johnston, Kushida, Stock, Rhoads and Stock, *ibid*, 1949, 2, 119.
44. Kidder and Dewey, *J. biol Chem*, 1949, 179, 181.
45. Elion, Burgi and Hitchings, *J. Amer. chem Soc.*, 1952, 74, 411
46 Hall, Richards, Willett and Feichtmeir, *Ann. N.Y. Acad Sci*, 1954, 60, 374.
47. Murphy, Tan, Ellison, Karnofsky and Burchenal, *Proc Amer. Assoc. Cancer Res*, 1955, 2, 36.
48. Farber, *Advances in Cancer Research*, Academic Press, New York, 1956, 4, p 43.
49. Agosin and Brand, *Antibiotics and Chemotherapy*, 1954, 4, 624.
50. Hewitt, Gumble, Wallace and Williams, *ibid*, 1954, 4, 1222.
51. Wright, Dolgopol, Logan, Prigot and Wright, *Proc. Amer. Assoc Cancer Res.*, 1955, 2, 55.
52. Taylor, Sherman and Bond, *J. Amer. chem Soc.*, 1954, 76, 4497.
53. Faiguenbaum and Alba, *Boletin chileno de parasitologia*, 1954, 9, 94
54. Timmis, Cooke and Spickett, *The Chemistry and Biology of Purines*, J. and A. Churchill, London, 1957, p. 148.

from one type of abnormal tissue to the other, we refrained from classifying histologically the malignant liver-parts, and shall refer to them as "liver tumours".

ANALYTICAL METHODS

(a) *Ashing of biological material.* Each group of pooled livers was weighed ("wet weight") and then dried in an electric oven at 110° for 24 hours. This material was powdered by grinding it in a glass mortar with glass pestle and dried at 110° to constant weight ("dry weight"). The powder was subsequently defatted by extraction in a soxhlet with absolute ether. The extracted residue was freed from solvent *in vacuo* with warming up to 50°. The product, weighed for "dry-defatted weight" ("W_{DD}") was incinerated in a "Vitreosil" crucible, placed in an electrically-heated muffle furnace at temperatures reaching 435° ± 1° inside 3 hours[15], when the heating was continued for another 7 hours. After cooling, the crucible was removed and its contents ground with an agate pestle. A few crystals of spectroscopically pure ammonium nitrate were mixed with the powder and the heating in the furnace continued at 435 ± 1° for 24 hours. The resulting colourless ash was weighed ("ash weight", "W_A") and stored in polythene specimen tubes until submitted to emission spectrographic analysis.

(b) *Emission spectrography.* The ash (*ca.* 20 mg.) was mixed with an equal weight of pure carbon powder and the mixture compressed into the bore of a Jelfke electrode made from spectrographically pure carbon rod to the following standard specification: external diameter of electrode, 3·0 mm.; internal diameter and depth of longitudinal bore, 0·8 mm. and 8·0 mm., respectively.

Four replicate spectrograms of each sample were obtained by the cathode layer arc technique (cf. Mitchell[14]) using a Hilger Large Quartz Spectrograph (E.492) and a 9 amp. D.C. arc. The external optical train consisted of a condensing·lens and step filter (ratio 1:1·7). The lens focussed the cathode layer on the collimator of the spectrograph, and the spectrograms were recorded on Kodak Photoscript B.10 plates over the range 2700 to 4800 Å. The samples were burned to completion in the arc; this required a 200 second exposure.

The wavelengths of the lines used for the estimation of each element are included in Table I. The density of the spectral lines on the sample plates were measured using a Hilger microphotometer, correction being made for "background" in the normal manner. The concentration of each element sought was estimated in a manner similar to that described by Mitchell[14], with the exception that Seidl density values[29] were used instead of normal density values, the concentration being read graphically from a working curve correlating log concentration in parts per million of ash (log W_{RI}) and log per cent relative density of the spectral line.

The mean working curves were established from the line densities on replicate spectrograms given by a series of synthetically prepared standards containing known quantities of the elements sought. Each standard was spectrographed four times.

CELLULAR CONSTITUENTS. MAJOR AND MINOR METALS IN NORMAL AND ABNORMAL TISSUES

PART I. ANALYSIS OF WISTAR RAT LIVERS FOR COPPER, IRON, MAGNESIUM, MANGANESE, MOLYBDENUM AND ZINC

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Assessment of the usefulness of emission spectrography for comparative analyses of six elements, Cu, Fe, Mg, Mn, Mo and Zn, in rat liver has been made. It has uncovered the presence or absence of differences in the concentrations of these metals in livers between age groups, animals on different diets, pregnant animals and male animals with normal, regenerating and tumourous organs. A discernible trend in concentrations of certain elements from the embryonic tissue to that of the adult animal emerged. In some instances the abnormal and regenerating materials appeared to carry lower or higher amounts of the element compared with normal livers. With molybdenum, the livers of embryonic, newborn and young animals had low concentrations, slowly increasing, the results obtained with older animals formed a higher level rugged plateau.

COMPARATIVE analyses of normal and abnormal tissues or cells for organic constituents and for levels of enzyme activities have been carried out over a number of years in many laboratories[1]. Estimation of nucleic acids, proteins, amino acids, lipids, carbohydrates, vitamins, coenzymes, hormones, etc. have been undertaken in embryonic, regenerating, adult and corresponding tumourous material. While so far usually only quantitative and not qualitative differences between the contents of various comparable tissues have been found, these observations are helping towards a greater understanding of the biochemistry of growth, development and cancer

More recent work has attempted to find more experimental support for the hypothesis[2] that induction of malignancy is due, at least in part to loss of, or change in essential proteins. Apart from the studies of the Millers[3] with azobenzenes, Weiler[4] from an immuno-chemical viewpoint established that the feeding to animals of butter yellow (4-dimethylaminoazobenzene) which leads to the development of hepatomas, causes also a loss of a liver antigen. He demonstrated later[5] a similar antigen loss in the kidney of the male hamster after insertion of stilboestrol pellets in the flank, producing over a period tumours[6] in that organ. Bhargava and Heidelberger[7] showed that 1 2·5 6-dibenzanthracene, a carcinogenic polycyclic hydrocarbon, in form of an oxidation product combined to a certain extent with the skin-proteins of mice which had been treated with the agent.

from one type of abnormal tissue to the other, we refrained from classifying histologically the malignant liver-parts, and shall refer to them as "liver tumours".

ANALYTICAL METHODS

(a) *Ashing of biological material.* Each group of pooled livers was weighed ("wet weight") and then dried in an electric oven at 110° for 24 hours. This material was powdered by grinding it in a glass mortar with glass pestle and dried at 110° to constant weight ("dry weight"). The powder was subsequently defatted by extraction in a soxhlet with absolute ether. The extracted residue was freed from solvent *in vacuo* with warming up to 50°. The product, weighed for "dry-defatted weight" ("W_{DD}") was incinerated in a "Vitreosil" crucible, placed in an electrically-heated muffle furnace at temperatures reaching 435° ± 1° inside 3 hours[13], when the heating was continued for another 7 hours. After cooling, the crucible was removed and its contents ground with an agate pestle. A few crystals of spectroscopically pure ammonium nitrate were mixed with the powder and the heating in the furnace continued at 435 ± 1° for 24 hours. The resulting colourless ash was weighed ("ash weight", "W_A") and stored in polythene specimen tubes *until submitted* to emission spectrographic analysis.

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Four replicate spectrograms of each sample were obtained by the cathode layer arc technique (cf. Mitchell[14]) using a Hilger Large Quartz Spectrograph (E.492) and a 9 amp. D.C. arc. The external optical train consisted of a condensing·lens and step filter (ratio 1:1·7). The lens focussed the cathode layer on the collimator of the spectrograph, and *the spectrograms were recorded on* Kodak Photoscript B.10 plates over the range 2700 to 4800 Å. The samples were burned to completion in the arc; this required a 200 second exposure.

The wavelengths of the lines used for the estimation of each element are included in Table I. The density of the spectral lines on the sample plates were measured using a Hilger microphotometer, correction being made for "background" in the normal manner. The concentration of each element sought was estimated in a manner similar to that described by Mitchell[14], with the exception that Seidl density values[29] were used instead of normal density values, the concentration being read graphically from a working curve correlating *log concentration in parts per million* of ash (log W_{gi}) and log per cent relative density of the spectral line.

The mean working curves were established from the line densities on replicate spectrograms given by a series of synthetically prepared standards containing known quantities of the elements sought. Each standard was spectrographed four times.

TABLE I

WAVELENGTHS OF LINES USED AND REPRODUCIBILITY OF RESULTS IN EMISSION SPECTROGRAPHY

Element and wavelength (A)	A		B	
	Known conc in standard (p p m)	Rel. standard deviation ± per cent	Mean conc. in sample (p p m)	Rel standard deviation ± per cent
Cu 3274	100	9 5	235	8 4
	316	8 4	386	6 6
	1000	2 8	509	7 8
	3162	6 5	1390	8 3
Fe 3059	1000	7 35	2560	21 1
	3162	7 98	6270	9 73
	10,000	6 54	7810	8 96
	31,620	7 05	20,200	10 89
	100,000	13 06	45,200	8 63
Mg 2783	1000	10 6	1040	15 1
	31,620	17 4	13,100	11 8
			16,000	9 9
Mn 2801	31 6	5 4	38	2 9
	100	4 5	55	5 8
	316	6 0	80	6 6
Mo 3170	3 2	15 8	7	13 8
	10 0	22 2	24	15 6
	31 6	11 4	34	18 8
	100	10 1	45	9 0
Zn 3345	3162	17 8	2450	12 1
	10,000	10 6	3250	10 9
			4830	12 7

The synthetic base, to which the elements were added in appropriate logarithmic proportions, was prepared by Messrs. Johnson and Matthey, London, by fusing the following mixture: KH_2PO_4 (21 0-g.); NaH_2PO_4 (4·96 g.); $CaCO_3$ (0·37 g.); Na_2CO_3 (0·42 g.); $NaNO_3$ (0·16 g.); NaCl (1·64 g.) and $(NH_4)_2SO_4$ (0·28 g.). The composition of this mixture was based on results of a bulk analysis of ashed rat organs, kindly undertaken by J. F. Harringshaw and L. S. Theobald of Imperial College, London, who found: —K_2O, 29·93; Na_2O, 8·19; CaO, 0·82; MgO, 2·15; Fe_2O_3, 0 04; SiO_2, 0 11; P_2O_5, 48·21; SO_3, 0·69; CO_2, 0·44; Al_2O_3, 0 02; N_2O_5, 0·4; NaCl, 2·97; H_2O, 1·02 per cent. Organic matter (containing N and/or O, 5·13 per cent); total, 100·12 per cent.

(c) *The reproducibility of the estimations* As previously mentioned, the present examination has been concerned only with the estimation of copper, iron, magnesium, manganese, molybdenum and zinc. Figures for the reproducibility of the results for those elements over the range of concentrations encountered are given in Table I.

Reproducibilities were estimated using both synthetically prepared standards (A) and the results obtained on selected samples (B). For the former, the reproducibility is given as the relative standard deviation obtained by expressing the standard deviation of four estimations as per cent of the known content of the standard. With the samples (B), the standard deviations are expressed as per cent of the mean of four replicate estimations.

The significance of these figures is that statistically the limits of the relative standard deviation should be exceeded only 1 in 3 times by

chance. Data A and B show relatively good agreement, and in view of the fact that the samples represented in B are those which showed maximum variation, it is considered that a satisfactory degree of reproducibility has been achieved with the spectrographic method employed.

Results and Discussion

In a preliminary spectrographic survey of 30 elements the following were not detected under our experimental conditions: Be, Bi, Cd, Co, Cs, Ga, Ge, Hg, In, Li, Sb and Tl. While Rb was a contaminant of the synthetic base (cf. p. 526), Ag, Ni, Sn and Zr were found to be present in small amounts in certain samples only, and Ba, Cr and Pb seemed to form genuine constituents of some of the livers. Our more extensive studies were restricted to six elements, namely, Cu, Fe, Mg, Mn, Mo and Zn, which are known to play an important role as part of enzymes or as their activators.

The results are reported in Table II and Histogram I, where by using different scales for the various metals and averaged values for some groups, the changes are visually summarised. The data are expressed as μg. of element per g. of dried defatted tissue (W_{DD}) or p.p.m., calculated from the ratio W_A/W_{DD} (as given in Table II, column 1) and multiplied by W_{g1} where $W_{g1} =$ μg. of element per g. of ash as obtained from the photometric values by the method mentioned above.

Copper (cf. Underwood[17], pp. 63–74). In normal livers the content increases suddenly from that of the embryos and newborn animals (cf. Lorenzen and Smith[30]) to about treble their amount in the one and two weeks old rats which are suckling at the time. The reason for this rise is rather obscure, particularly if one considers the low copper content of milk reported in the literature[31]. Our observation confirms that of Brückmann and Zondek[32], who have found a peak in copper concentrations in rat livers between 10 to 15 days after birth. Like these authors we established that in our animals, fed on a controlled diet, the copper content declined during the period from the 3rd to 16th week. Animals, 4 to 8 weeks old and pregnant rats 16 to 20 weeks old, both groups fed on kitchen scraps, did not differ very much from the former (cf. Ashikawa, Smith and Helwig[33]). The "liver tumours" from rats over 12 months old and the regenerating livers, after 5 and 8 days regeneration show only a slight increase compared with the organs of all adult animals mentioned above.

Iron (cf. Underwood[17], pp. 26–33). The level in tissues of this major element is, for obvious reasons, subject to fluctuations by the presence or absence of residual blood. Attention is therefore drawn only to the higher values noted in foetal livers of embryos and newborn animals, in liver tumours and in the regenerating organ. The average iron content of all other livers (21 groups) given in Table II is 236 p.p.m.

Magnesium. Figures for this other major element, so far obtained, are of restricted significance without the corresponding data for calcium. However, the values for the regenerating organ seem to be genuinely lower and the average for the liver tumours (986 p.p.m. from 7 samples) appear to be higher than the average of all other livers, namely 627 p.p.m.

TABLE I

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	31 6	11 4	34	18 8
	100	10 1	45	9 0
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NaCl, 2·97; H_2O, 1·02 per cent. Organic matter (containing N and/or
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standard deviations are expressed as per cent of the mean of four replicate
estimations.

The significance of these figures is that statistically the limits of the
relative standard deviation should be exceeded only 1 in 3 times by

TABLE II

INDIVIDUAL ANALYTICAL RESULTS OF ALL SAMPLES

Age	Ratio W/W_{Zn}	Copper p.p.m in ash	Copper p.p.m in sample	Iron p.p.m in ash	Iron p.p.m in sample	Magnesium p.p.m in sample	Magnesium p.p.m in ash	Manganese p.p.m in ash	Manganese p.p.m in sample	Molybdenum p.p.m in ash	Molybdenum p.p.m in sample	Zinc p.p.m in ash	Zinc p.p.m in sample
Foetal	0·056	28·8	599	16,400	928	776	13,700	32	1·81		0·17	4260	241
..	0·077	31·1	534	8,540	658	847	13,400	33	2·02	2	0·17	3770	271
..	0·067	29·7	470	6,600	417	713	11,300	33	2·08	2	0·14	3370	215
..	0·072	25·2	516	6,150	441	673	9,380	35	2·15		0·13	4100	308
New born	0·047	32·9	599	12,300	531	595	12,600	35	1·92	4	0·22	6510	302
..	0·035	32·9	1390	10,150	557	540	9,800	73	1·91	17	0·98	3510	255
1 week	0·059	35·2	1150	6210	363	632	10,800	73	2·14	30	1·47	4350	223
2 weeks	0·037	31·2	588	4730	145	752	13,800	79	1·90	35	1·70	4150	159
3 weeks	0·050	29·5	386	2560	143	679	13,000	73	3·87	46	1·80	2820	119
4 weeks*	0·049	19·6	262	2850	222	650	13,300	73	2·96	40	2·15	2470	101
5 weeks*	0·051	13·4	205	3170	210	780	15,200	78	2·98	45	1·62	2430	69
6 weeks*	0·041	11·5	253	5030	261	591	14,600	69	3·45	43	2·30	1980	97
8 weeks*	0·045	23·5	232	8390	379	760	12,100	67	3·94	34	1·73	1960	93
16 weeks*	0·032	8·8	228	3450	126	551	10,700	79	3·65	34	1·44	1890	80
4 weeks†	0·038	9·5	215	5190	172	492	13,100	65	2·88	38	1·63	1930	100
6 weeks†	0·046	10·0	207	5800	240	605	13,100	82	3·88	28	1·23	3240	100
5 weeks†	0·043	10·2	191	5530	288	547	12,200	82	4·00	32	2·04	2100	65
8 weeks†	0·049	9·1	268	3750	199	699	12,000	86	4·35	39	1·97	1700	103
8 weeks†	0·051	9·7	212	6140	238	633	11,800	84	4·06	37	2·08	1510	106
8 weeks†	0·031	15·2	301	4050	186	996	11,300	68	2·10	34	1·52	2130	114
8 weeks‡	0·048	11·0	229	2110	295	705	12,600	73	3·45	34	1·56	2290	143
16–20 weeks§	0·046	18·7	157	3110	301	520	12,500	76	3·45	13	1·92	2970	97
5 days**	0·051	11·4	240	29,600	1630	571	3970	44	1·97	15	Nd	2110	95
8 days**	0·052	12·9	273	9940	461	237	4100	40	2·28	11	0·90	2060	92
..	0·055	12·8	131	3800	1050	245	14,700	30	3·13	18	1·14	Nd	Nd
12–14 months**	0·096††	13·2	147	13,800	365	1120	13,900	57	3·91	23	1·80	1840	127
..	0·055	19·0	220	8810	363	763	15,700	76	2·91	24	0·61	Ca	Nd
..	0·035			9770	345	799	14,200	40	2·91		1·80	2750	130
..	0·047			4345	143	1190	15,200	41	2·95		0·29	2620	133
..	0·091			8880	803	1350	14,900		1·72		2·18	Ca	High

* Rats were fed on a controlled diet.
† Rats were fed on a normal diet.
‡ Rats were fed on a normal diet plus kitchen scraps and the livers were pooled (four).
§ Maternal livers from pregnant rats

Regenerating livers
** Tumours arising from rats fed on a diet containing a carcinogen
†† Perfused with sodium citrate.

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(522)

HISTOGRAM 1. Individual analytical results of samples.

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REFERENCES

1.   Ioeber,
2.
3.   York.
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8.
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10.
11.   cf. also Bergel, f Purines, Ciba
    Foundation Symposium, 1957, 256, J. and A. Churchill, London.
12. Ambrose, James and Lowick, Nature, Lond., 1956, 177, 576.
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Manganese (see Underwood[17], pp. 235–240). An increase in content of this metal can be discerned (see Histogram I) from the embryo via the newborn and one to two weeks old animals to the three to 16 weeks old rats. It may be significant that the values for the regenerating livers are more like those of the earlier age groups, while those of the liver tumours are similar to the results with the later age groups.

Molybdenum (see Underwood[17], pp. 125–129). This trace element is of special interest to us in view of the work in these laboratories[9] on xanthine oxidase, a molybdeno-flavoprotein. The low levels of foetal and newborn livers rise over the next five weeks and arrive at an average value for the 16 weeks old, the pregnant females and the rats fed on kitchen scraps of 1·7 p.p.m. This should be contrasted with the content of the liver tumours of which three showed a low molybdenum concentration and four an average of 2·0 p.p.m. It has been mentioned before that the histological nature of the tumours was somewhat uncertain. This might explain the differences between the low and higher figures. The low values could be quoted in support of a report by Westerfeld and colleagues[8] on the diminished level of xanthine oxidase in livers of animals fed with butter yellow. Our figures obtained with foetal livers invite a similar comparison with the low level of xanthine oxidase activity in embryonic organs[34]. No molybdenum could be detected in our regenerating livers. If this is an observation reproducible with other material, it should be followed up by estimation of molybdeno-flavoproteins in regenerating organs.

Zinc (*cf.* Underwood[17], pp. 208–216). Our figures suggest a higher zinc content in the foetal, newborn and one week-old livers. Then they fall to an average of 102 p.p.m. in the organs of animals between 2 and 16-weeks old, organs with the data for all the other normal livers being about the same[35]. We cannot offer, at present, any explanation for the apparent absence of zinc in the regenerating livers. Some of the tumour livers did not yield results, because the presence of surprisingly high amounts of calcium made the spectrographic estimation of zinc impossible. This observation makes it very desirable to analyse at an early date for calcium. The remainder of the tumour-livers showed slightly higher metal contents, average 130 p.p.m. The other point which should be mentioned here, is that the keeping of rats in zinc cages has seemingly very little effect on the content of zinc in the livers

There is no doubt that further work has to aim at the inclusion of other elements including major ones, such as sodium, potassium and calcium and to be backed up with coenzyme enzyme determinations or assessment of other metal-bearing cellular constituents Future studies should be extended to cellular particles and to other organs in the same species and to organs of other species, finishing in the end with comparable human material.

sized (Timmis, Cooke and Spickett, 1956). Known methods have been adapted and used fairly extensively. Thus, 2 : 6-dichloro-5-nitro- or 2 : 6-dichloro-4-methyl-5-nitropyrimidine (IV, R^3 = H or Me) was cautiously treated with benzylamine, furfurylamine, cyclohexylamine or 2-amino-6-methylpyridine to yield the corresponding 2-chloro-6-pyrimidylamine (V). From this intermediate stage, purines and 8-azapurines containing a chlorine substituent in the 2-position (II and III, R^1 = Cl) were derived, by reduction with hydrogen and Raney nickel to the diamine (VII) and treatment of this with formamide at 180° and nitrous acid respectively.

R = H, Me, Cl R^1= Cl, SH, NH_2 R^3= H, CH_3

R^2= benzyl, furfuryl, cyclohexyl, 2- pyridyl, 6- methyl-2- pyridyl.

For the synthesis of the corresponding 2-amino derivatives, the appropriate 2-chloro-6-pyrimidylamines (V) were treated with a hot alcoholic solution of ammonia to yield (VI) from which the diamines (VII) were obtained by reduction with stannous chloride or with hydrogen and Raney nickel. The final ring closures were again effected with formamide at 180° and with nitrous acid. Where R^2 in (II) and (III) was 6-methyl-2-pyridyl-, a mercapto group was introduced at the 2-position (R^1) by treatment of the appropriate 2-chloro-6-pyrimidylamine (IX, R = H or Me) with boiling aqueous ammonium sulphide solution whereby reduction of the nitro

SYNTHETIC CHEMICAL INVESTIGATIONS
RELATED TO THE METABOLISM OF PURINES

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THE work which I wish to describe was concerned, from the
practical point of view, with the search for new drugs active
against acute leukaemia and possibly other malignancies, and
also in a more academic way with the eventual study of purine
metabolism through the preparation of new types of anti-
purines and the synthesis of purine-N-oxides, which might
possibly represent a step in the *in vivo* oxidation of purines.

Purine itself and certain simple derivatives (I, R = H, Me,
Cl, SH) are antipurines (antiadenine or antiguanine) and are
effective in certain experimental leukaemias or on sarcoma
180 (Clarke *et al.*, 1954). With the exception of 6-mercapto-
purine, however, they are not successful clinically because of
their toxicity. Since in the biosynthesis of nucleic acid the
ribose moiety is attached to a precursor of the purine and
remains attached at the 9-position of the purine moiety
throughout the synthesis, it seemed that, on the basis of
structure analogy, a sugar residue might be advantageously
introduced into the structure of potential antipurines at the
appropriate position; also, toxicity might be lessened by this
modification. However, recent work (G. B. Brown, this
symposium) has revealed an unexpected enhancement of
toxicity, probably attributable to phosphorylation of the
ribose, when the ribosides of purine and 6-methylpurine are
compared with the parent purines. These findings suggested
that, instead of the sugar residue, somewhat similar cyclic but
non-hydroxylic substituents should be tried. A variety of new
purines and 8-azapurines (II and III) were therefore synthe-

Me) with hot formic acid and final ring closure with warm dilute sodium hydroxide solution. The 8-azapurines were formed by reaction with the calculated quantity of nitrous

group and replacement of chlorine by the mercapto group
yielded the diamine (XI). Alternatively (IX, $R^3 = H$) on

treatment with thiourea in boiling ethanol yielded the 2-*iso*-
thioureido derivative (X) from which (XI, $R^3 = H$) was
obtained on reduction with ammonium sulphide.

The purines were obtained from (XI) either directly by
treatment of the hydrochloride with formamide at 180°
or by intermediate formation of the formamido derivative
(VIII, $R^1 = SH$, $R^2 = $ 6-methyl-2-pyridyl-, $R^3 = H$ or

fact attached to the 3-nitrogen atom rather than the 2-carbon atom (a conceivable but unlikely alternative) was indicated by benzoylation of the tautomeric form (5-amino-1-hydroxy-2-phenyl-1 : 3 : 4-triazaindene) when the dibenzoyl derivative on infrared examination showed the presence of a band at 1783 cm.$^{-1}$ indicating an $= N \cdot O \cdot CO \cdot Ph$ group, and not a $= C \cdot O \cdot CO \cdot Ph$ group at the 2-position which would have shown a band at about 1725 cm.$^{-1}$ The 6-benzamido group was indicated by the expected band at 1676 cm.$^{-1}$ From the reaction of 4-amino-5-nitroso-pyrimidines (XX) with aryl aldehydes it was not possible to isolate any purine-N-oxides, but the use of the aldehyde-anils led to very rapid formation of the required products (XXII). Presumably their relatively slow formation in the first case permitted their transformation to other products whilst considerable amounts of nitroso compound remained unattacked. Although the postulated intermediate (XXI) could not be isolated it seems likely that the initial attachment of the aldehyde would be to the amino group rather than to the nitrogen of the nitroso group (in the oxime form) since it is shown below that acylation with benzoic anhydride or benzoyl chloride occurs on the amino group.

The facile preparation of 6-amino-2-aryl-1 : 3 : 7-triazaindene-3-N-oxides from aryl aldehydes and 2 : 6-diamino-3-nitrosopyridine is probably due to the speed of formation of the intermediate azamethine and to the relative stability of the N-oxide in the acetic acid medium used.

A property of the 8-arylpurine-7-N-oxides so far made is the remarkable instability of the iminazole ring towards treatment with weak acids. Thus 2 : 6-diamino-8-phenylpurine-7-N-oxide, on boiling for a few minutes with acetic acid, gave in good yield equimolar quantities of (XXV) and (XXVII). The most probable mechanism would involve the initial formation of the hydroxylamine (XXIII), by hydrolytic fission of the iminazole ring, followed by disproportionation to yield (XXV) and (XXIV) which under the conditions of the experiment would readily ring-close to yield (XXVII). These results are also consistent only with the oxygen being attached

acid at 0°. If the reaction was carried out with any excess of
nitrous acid or at a higher temperature the predominant
product was the corresponding $2 : 2'$ bis-azapurinyldisulphide
(XVIII). In fact, the best method found for oxidizing the
2-mercapto-azapurines to the corresponding disulphides was
treatment with nitrous acid.
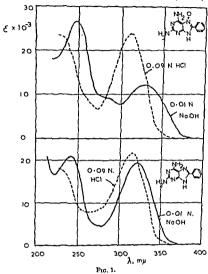
Where $R^2 =$ 2-pyridyl a very convenient new route to (II)
and (III) was afforded by treating e.g. $2 : 6$-dichloro-4-methyl-
5-nitropyrimidine with 2-aminopyridine to yield (XII,
$R^3 =$ Me); this led, on treatment with hot methanolic am-
monia solution to (XIII, $R^3 =$ Me) (Spickett and Timmis,
1955), or by treating with hydrogen sulphide in cold aqueous
solution to (XIV, $R^3 =$ Me) and thence to the purine and
azapurine.

Compounds of the two series were tested on a purine-
requiring strain of *Esch. coli* by Collier and Huskinson (this
symposium) who found that (XV), (XVI), (XVII) and (XIX)
(R = H, Me) showed activity as antiguanines. The degree of
competitive effect varied from compound to compound. (XV)
was competitive over a fairly good range and comparison
showed it to be about three times as active as 6-mercapto-
purine, as an antiguanine. This compound, therefore, and
others of the two series will be investigated further in experi-
mental animals.

It was suggested at a Ciba Foundation Symposium (Timmis,
Felton and Osdene, 1954) that purine-N-oxides would be
worth making as potential antimetabolites. Also, although
knowledge of the mechanism of action of xanthine oxidase
in the oxidation of purines argues against any possibility of
an N-oxide being an intermediate structure in these cases,
the possibility perhaps remains that the oxidation of, e.g.
inosinic acid which is not catalysed by xanthine oxidase,
might involve N-oxide formation.

In preliminary synthetic experiments reported previously
(Timmis, Felton and Osdene, 1954) benzaldehyde and $2 : 6$-
diamino-3-nitrosopyridine reacted to yield 6-amino-2-phenyl-
$1 : 3 : 7$-triazaindene-3-N-oxide. That the oxygen was in

to (XXVII) is very facile, unlike the ring closure of 4-amino-
5-benzamido-pyrimidines (cf. Elion, Burgi and Hitchings,
1951), was proved by the reduction of (XXV) to (XXIV) with

FIG. 1.

an aqueous solution of sodium dithionite at room temperature
and warming (XXIV) in acetic acid when (XXVII) was obtained
in good yield.

Fig. 1 shows ultraviolet absorption spectra curves for

to the nitrogen in the 7-position and not to carbon in the
8-position. Confirmation is afforded by analogy with the con-
clusion drawn from the infrared examination of dibenzoyl-
5-amino-1-hydroxy-2-phenyl-1 : 3 : 4-triazaindene. 5-Nitroso-
2 : 4 : 6-triaminopyrimidine on benzoylation yielded (XXV).
That the benzoyl group was attached to the 4-(or 6-)amino
group was proved by infrared examination, which showed a
band at 1653 cm.$^{-1}$, clearly indicating an $- NH \cdot CO \cdot Ph$
group, and ruling out the alternative structure (XXVI) where
a band at not less than 1760 cm.$^{-1}$ would have been shown for
the $= N' \cdot O \cdot CO \cdot Ph$ group.

$R^1 = NH_2$, 5Me, $R^2 = Ph$, $C_6H_4 OMe (4)$, $C_6H_4 Cl (4)$, $C_6H_3Cl_2 (3 4)$, piperonyl.

Confirmation of structures follows from the other reactions
summarized in (XX) to (XXIX). That the step (XXIV)

ENZYMIC CONTROL OF PURINES BY
XANTHINE OXIDASE

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I WISH to present, in the name of·my colleagues, Bray, Haddow and Lewin, and my own, a brief review—though very incomplete—of the possible rôle of xanthine oxidase as an enzyme that controls the general pool of biological purines by catabolizing hypoxanthine and xanthine to uric acid. Deeply interested in the part played by quantitative differences of cellular constituents in the phenomena of cytological normality and abnormalities, we accept at present the view that the existence of a special pattern (Greenstein, 1954) of, say, functional proteins and nucleotides more than just excess or deficiency of individual components, may be behind the puzzle of malignancy. Nevertheless, we speculate that certain enzymes, while integrated with other catalytic systems into a decisive whole, hold key positions, so that their quantitative changes in some organs and tissues may be a reflection or even a cause of profound cellular upheavals.

As indicated above, we have made the assumption that xanthine oxidase is such a key enzyme. From recent work by Abrams and Bentley (1955 *a* and *b*) and others, there emerges the picture of a "purine cycle" (Table I, cf. Feigelson and Davidson, 1956) which consists of mutual transformations of purine nucleotides, nucleosides and bases. I do not wish to go into details, but I should like to point out two or three pertinent features: apart from the formerly established deaminases, enzyme systems have been discovered in rabbit bone marrow (Abrams and Bentley, 1955 *a* and *b*) which are responsible

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2 : 6-diamino-8-phenylpurine and its 7-N-oxide, indicating the appearance of a new anionic centre in the N-oxide, in alkaline solution.

REFERENCES

Clarke, D. A., Phillips, F. S., Steinberg, S. S., and Stock, C. C. (1954). *Ann. N.Y. Acad. Sci.*, 60, art. 2, 235.

Elion, G. B., Burgi, E., and Hitchings, H. G. (1951). *J. Amer. chem. Soc.*, 73, 5235.

Spickett, R. G. W., and Timmis, G. M. (1955). *J. Chem. Soc.*, 4354.

Timmis, G. M., Cooke, I., and Spickett, R. G. W. (1956). *J. chem. Soc.*, in press.

Timmis, G. M., Felton, D. G. I., and Osdene, T. (1954). Ciba Foundation Symposium on the Chemistry and Biology of Pteridines, p. 93. London: J. & A. Churchill Ltd.

Fig. 1. Photomicrograph of crystals of purified xanthine oxidase × 800.
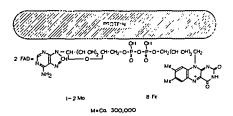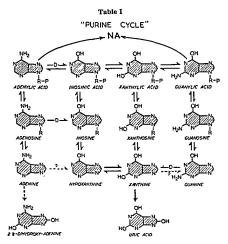
Fig. 2. Composition of xanthine oxidase.

for amination, in presence of glutamine and a phosphate energy source, of xanthylic acid to guanylic and inosinic acid —which is the *de novo* entry from purine precursors (Greenberg, 1956; Buchanan, 1956)—to adenylic acid. More important still, an inosinic acid dehydrogenase has been found which

Table I

"PURINE CYCLE"

is not identical with xanthine oxidase (*it does not affect hypoxanthine*), but is dependent on DPN. Thus, in addition to xanthine oxidase there exists a second oxidation system, which seemingly occurs not only in mammals but also in bacterial cells (*Aerobacter aerogenes, Esch. coli*) (Gehring and Magasanik, 1955; Magasanik, 1956), although the situation there may be

Table II

RATES OF OXIDATION OF VARIOUS SUBSTRATES CATALYSED BY XANTHINE OXIDASE

Symbols a) Substrates X = xanthine, H = hypoxanthine   A = acetaldehyde   D = DPNH
b) Electron acceptors O = oxygen Mb = methylene blue, I = 2 6 dichlorophenol-indophenol, Cy = cytochrome c

Reaction Sample	Absolute Rates			Rates as % of corresponding X/O rate					
	X/O	H/Mb[1]	A/Mb[1]	X/O	X/I	A/I	O/I	O/O	H/Cy
Corran et al	.	.	.	.	.	.	.	.	.
Morris [2]	0 52	.	.	.	.	.	.	.	.
Mackler et al [1]	11 29)	.	.	(1 90)(2 90)(1 69)	(10)	(50)	6	30	
LXO56 MT	1 82	1 5 2 3[b]	.	.	.	.	.	.	.
LXO60 MT (freshly prepared)	1 68	1 1 5 9[4]	2 6	100	41	12	.	8 7	.[5]
" " (after 9-4 weeks)	0 8[6]	.	.	150	41	15	1 5	.	6 1[5]
LXO60 MS	0 93	.	.	100	41	18	1 5	8 7	6 1[5]
LXO61 M6	0 95	.	.	156	42	20	1 9	6 9	6 1[5]
LXO63 M1 (Buttermilk)	0 92	.	.	100	29 .	18	2 6	1 5	.
LXO65 Whole milk	.	.	.	100	.	.	6 4	.	.
LXO60 "inactive" sample (ex M7)	0 005	.	.	100	.	18	600	200	.

Absolute rates are moles substrate oxidised per mg protein per minute   Figures in parentheses are
extrapolations by the Lineweaver and Burk method
    [1] Conditions as given by Corran et al   (4) Obtained with a reduced hypoxanthine
    [2] pH 8 5, 15°C   concentration
    [3] pH 7 0, 22°C   [5] Identical figures were obtained whether
                                                       NaCN was present or absent

points: the activities of our pure xanthine oxidase preparation
with DPNH = D as substrate and cytochrome c = Cy as
acceptor, are relatively low, and the sample LXO60 is in-
effective only in catalysing oxidation of purines and aldehyde
but is still capable of dehydrogenating DPNH. An interesting
discovery of another group of electron acceptors was made by
our colleagues Ross and Warwick (1955), when they found
that some compounds possessing the azobenzene structure
(Fig. 8) were reduced, though at low rates, by the system
xanthine oxidase-xanthine. In order to obtain an indication
of whether this system would act as a reducing agent under
biological conditions on carcinogenic derivatives of azobenzene
we tested it on butter yellow. With this dyestuff, dispersed
with the help of an anionic detergent, in an aqueous medium
no measurable reaction could be recorded. In this connection

more complex. It links the 6-hydroxypurine column with that of the 2 : 6-dihydroxy series. But this does not alter the fact that, allowing for the limits of present-day knowledge, the sole biochemical exit or leak is that promoted by xanthine oxidase in catalysing the hydroxylation of xanthine to uric acid, a reaction which in contradistinction from hypoxanthine-xanthine transformations has never been shown experiment-ally to be reversible. I should add that Klenow (1952) has demonstrated the oxidation of adenine by xanthine oxidase to 2 : 8-dihydroxyadenine.

Before quoting a number of experimental results which seem to support the hypothesis that xanthine oxidase exerts a con-siderable influence on the overall equilibria of all the reactions given in Table I (cf. Feigelson and Davidson, 1956), I should like to give a short resumé of the chemistry of xanthine oxidase. Although there is no definite evidence yet, the identity or close similarity of the holo-enzyme in liver and other tissues of various mammalian species, with that from cows' milk from which, among others, we have prepared our concentrates, can be assumed. While Kielly (1955) has investigated relatively crude preparations from calf's liver, our crystalline material (Avis, Bergel and Bray, 1955; Avis et al., 1956) (Fig. 1), showed a composition (Fig. 2) very similar to that given by her. There is the large proteinous part with 2 FAD, 8 Fe and a somewhat changeable content of 1-2 Mo with a total M of ca. 300,000. Even our best fully crystalline samples still consist of a mixture of active and physicochemically closely related inactive molecular species, the separation of which we are studying at present. So far, we have succeeded in obtaining an inactive, fully crystalline metalloflavoprotein which was chemically indistinguishable from the enzymically potent samples. The importance of this will emerge in a few moments. First let us glance at Table II (Avis, Bergel and Bray, 1956) which gives rates of oxidation of various substrates, X = xanthine, H = hypoxanthine, A = aldehyde in presence of oxygen or electron acceptors such as Mb = methylene blue, I = dichlorophenol-indophenol. May I draw attention to two

line-sulphonic acid, glutathione, etc. protected only to a very
small extent.

This finding has enabled us to make a start with storing
larger amounts of enzyme for a full scale biological trial to
confirm or otherwise the preliminary results by Haddow,
namely that injection of xanthine oxidase concentrates into
mice of various strains, carrying spontaneous mammary
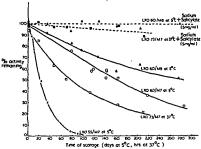
FIG. 4. Stabilization of xanthine oxidase by sodium salicylate.

carcinoma (Fig. 5), arrested the growth of the tumours.
Moreover, the preparation of an inactive, or, as Frieden (1956)
called it recently in the lysozyme field, "enzymoid" metallo-
flavoprotein, physicochemically not very different from the
fully effective one, should enable us to carry out a complete
set of experiments, ascertaining that the antigrowth effect on
the mammary tumour is either due to the specific action of
the enzyme or to an unspecific action of the non-functional
flavoprotein. I am not going to argue away at this juncture
the problem of cell-membrane penetration by a giant molecule
of 300,000 !

it is interesting to recall the findings of Westerfeld, I
and Hilfinger (1950), namely that administration of
yellow to rats decreases the xanthine oxidase activity c
kidney and blood.

The instability on storage of purified xanthine c
samples made their chemical or biological use, up to a
date, rather difficult. The cause of the diminution of a
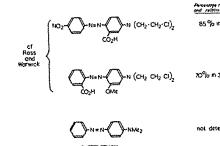
Fig. 3. Azobenzene derivatives as electron acceptors of the xanthine c
xanthine system.

which occurs with different samples at different rate
which, as said before, does not always include that t
DPNH, is still unknown. It may be due to loss of M
slight changes in the protein structure or in the FAD.
matic investigations by my colleague Bray have led, ho
to the discovery that added sodium salicylate inl
deterioration of the oxidase and dehydrogenase act
(Fig. 4). Salicylate proved to be the best stabilize
aspirin, PAS, benzoic acid, uric acid, etc. still exerted n
able protection, while salicylamide, versene, 8-hydroxy

It is of some significance that administration of one of the substrates, xanthine, to mice in short-term (14–20 days) experiments by Feigelson, Feigelson and Wood (1954) has produced elevation of xanthine oxidase levels and of adenosine deaminase in the liver (Table III), while its administration to

Table III
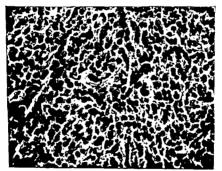
THE EFFECT OF XANTHINE ADMINISTRATION ON THE CONCENTRATIONS OF LIVER XANTHINE OXIDASE AND ADENOSINE DEAMINASE (FEIGELSON, FEIGELSON AND WOOD, 1954)

Group No	I	II	III
Xanthine administered (mg/day, per 100 g body weight)	0	12	24
Xanthine oxidase			
μlO₂/hr, per g wet weight	342(321-361)	422(382-438)	493(435-614)
μlO₂/hr, per g dry weight	1360(1180-1570)	1850(1480-2010)	2110(1780-2940)
μlO₂/hr, per μM nitrogen	0.163(0.148-0.181)	0.208(0.177-0 230)	0.236(0.187-0.313)
Adenosine deaminase			
μM adenosine deaminated/hr, per g wet weight	46.9(28.1-69.8)	61.6(51.2-69.7)	86.6(64.0-141.1)
μM adenosine deaminated/hr, per g dry weight	185(117-245)	272(201-323)	301(263-672)
μM adenosine deaminated/hr, per μM nitrogen	0.0226(0 0136-0.0345)	0.0308(0.0241-0 0403)	0 0416(0.0288-0.0716)

rats, in long-term experiments by Haddow, gave rise to granulomata of which a proportion developed into malignant sarcomas. (Fig. 6 shows a microscopic picture of tumour tissue, published by courtesy of E. Horning.) So far, esti- mations of enzyme levels in the latter series have not been carried out; it would be most interesting to see whether prolonged injections cause, after an initial rise, a final fall in xanthine oxidase content in the rat tissues. There is another situation in which, due to an extraneous factor, namely virus infection, xanthine oxidase activity rises; Bauer (1948) found such increase in mouse brain during invasion by different viruses, and Sellers and Jann (1954) came to a similar conclu- sion (2–2·5 × XO) with respect to mouse lungs infected with influenza virus, the enzyme assays confirmed by uric acid estimations. An immediate interpretation of these observa- tions concerning nucleic acid synthesis by the host for the

Fig. 5. Mouse (C+ strain) with spontaneous mammary carcinoma.
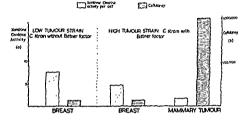
Fig. 6. Photomicrograph of a spindle cell sarcoma, induced by xanthine in a rat.

(d) On the other hand, breast tissue of C+ mice during lactation contains 5–6 times as much xanthine oxidase as that of adult virgin mice of the same strain. This is exclusive of the xanthine oxidase of the milk in the lactating mouse, as the new technique of assay eliminates the contribution of the residual milk.

Table IV

Xanthine Oxidase During Carcinogenesis in Mouse Breast

1 The enzyme activity per mammary cell increases during normal growth of breast

2 The enzyme activity per mammary cell is lower in the cancer-susceptible than in the cancer resistant mouse

3 The enzyme activity per tumour cell is very low in comparison to the breast cell Whether this fall in enzyme content per cell is associated with a fall in enzyme concentration within the cell is under study

(a) = moles [$\times 10^9$] xanthine oxidized/$\times 10^9$ nuclei/min.

(b) Nuclei/mg. wet tissue

Two points are deserving of consideration. First, it appears that normal growth which leads to the lactating stage is associated with a rise in xanthine oxidase, while tumour growth goes hand in hand with a decrease. Secondly, and this may alter somewhat the overall conclusions, tumour cells in comparison with normal cells usually possess larger nuclei

virus cannot be given yet, but it is felt that it has some direct or indirect bearing on the key rôle of xanthine oxidase, particularly if one takes into account what Greenberg (1956) and Buchanan (1956) had to say on enzymic synthesis of inosinic acid, and if one remembers Table I.

There is one series of results, however, obtained by our colleague Lewin, which we think gives further proof of the validity of our working hypothesis. Lewin has carried out a systematic investigation into the xanthine oxidase content of breast tissue of female mice in different states of the reproductive cycle, mice belonging to the C+ and C− strains; that is, with and without the so-called Bittner factor, or high and low cancer strain. In the first instance, he worked out a reliable method of assay based on Kalckar's (1947) method and consisting of spectrophotometric measurements of the rate of appearance of uric acid from xanthine, after tissue homogenates had been subjected to high-speed centrifugation and subsequent incubation. This new method has certain advantages over the hitherto applied one which rests on the reduction of dyestuffs, such as dichlorophenol-indophenol. Lewin produced a great number of results which can be summarized as follows (Table IV):

(a) Breast tissues from virgin mice, mated mice that had not yet developed tumours, and from mated mice that had developed tumours, all of the C+ strain, showed no significant difference in xanthine oxidase activities per cell.

(b) Mammary tumour tissue of the same strain had, per cell, a significantly lower level of enzyme activity.

(c) When the non-tumorous breast tissue of the C+ mice, which one can assume are in a pre-cancerous state, were compared with breast tissue from C− mice, it was found that the latter was richer in xanthine oxidase per cell. If this activity is taken as 100, the activity for C+ breast tissue is 54 and that for the tumour 24 on a cellularity basis.

REFERENCES

ABRAMS, R., and BENTLEY, M. (1955a). *Arch. Biochem. Biophys.*, 56, 184; 58, 109.

ABRAMS, R., and BENTLEY, M. (1955b). *J. Amer. chem. Soc.*, 77, 4179.

AVIS, P. G., BERGEL, F., and BRAY, R. C. (1955). *J. chem. Soc.*, 1100.

AVIS, P. G., BERGEL, F., and BRAY, R. C. (1956). *J. chem. Soc.*, 1219.

AVIS, P. G., BERGEL, F., BRAY, R. C., JAMES, D. W. F., and SHOOTER, K. V. (1956). *J. chem. Soc.*, 1212.

BAUER, D. J. (1948). *Nature, Lond.*, 161, 852.

BUCHANAN, J. M. (1956). This Symposium, p. 233.

FEIGELSON, P., and DAVIDSON, J. D. (1956). *Cancer Res.*, 16, 352.

FEIGELSON, P., FEIGELSON, M., and WOOD, T. R. (1954). *Science*, 120, 502.

FRIEDEN, E. H. (1956). *J. Amer. chem. Soc.*, 78, 961.

GEHRING, L. B., and MAGASANIK, B. (1955). *J. Amer. chem. Soc.*, 77, 4685.

GREENBERG, R. (1956). This Symposium, p. 204.

GREENSTEIN, J. P. (1954). Biochemistry of Cancer, 2nd edn., New York: Academic Press.

KALCKAR, H. M. (1947). *J. biol. Chem.*, 167, 429.

KIELLY, R. K. (1955). *J. biol. Chem.*, 216, 405.

KLENOW, H. (1952). *Biochem. J.*, 50, 404.

MAGASANIK, B. (1956). *J. Amer. chem. Soc.*, 78, 1510.

REID, E., O'NEAL, M. A., and LEWIN, I. (1956). *Biochem. J.*, 64, 33.

ROSS, W. C. J., and WARWICK, G. P. (1955). *Nature, Lond.*, 176, 298.

SELLERS, M. I., and JANN, G. J. (1954). *Proc. Soc. exp. Biol. N.Y.*, 86, 205.

VILLELA, G. G., MITIDIERI, E., and AFFONSO, O. R. (1955). *Nature, Lond.*, 175, 1087.

WESTERFELD, W. W., RICHERT, D. A., and HILFINGER, M. F. (1950). *Cancer Res.*, 10, 486.

and less of the cytoplasmic mass. As nuclei appear to be devoid of xanthine oxidase (Villela, Mitidieri and Affonso, 1955), and this is supported by Lewin's own observations, it is feasible that in consequence there is a higher concentration of xanthine oxidase in the tumour cytoplasm, although there is less enzyme available for the whole cell. Further studies should clarify this situation.

When estimating xanthine oxidase in mouse livers, alteration in hormonal balances associated with pregnancy and lactation do not affect the enzyme levels. But when our colleagues Reid, O'Neal and Lewin (1956) extended these assays to livers of rats which had been submitted to hypophysectomy or adrenalectomy, the following interesting picture emerged: subsequent to the former, xanthine oxidase activity went up and the apparent rate of cytoplasmic RNA synthesis and accumulation fell while, following adrenalectomy, the opposite effects were observed.

Summary

May I finish on the optimistic note produced by these results. Of course the situation may be very much more complex than one is hoping for at this moment. Apart from xanthine oxidase a great number of other enzyme systems could be involved. But the preliminary results presented here, namely arrest of spontaneous mammary tumours by xanthine oxidase concentrates, the differences between various mammary tissues in xanthine oxidase contents, the newly discovered rôle of some azobenzenes as electron acceptors for the xanthine oxidase-xanthine system and the apparent importance of the enzyme for virus synthesis make it worthwhile, I think, to pursue the working hypothesis of xanthine oxidase as a key enzyme controlling the purine pool and thus NA synthesis, and to continue with chemical elucidation of this flavoprotein which, for me as an organic chemist, is nearest to my heart.

body, is readily accessible. It is clear that an understanding of morphogenesis in any particular instance will be greatly advanced when, by means of microscopy, the fine structures of the cells involved and the structural aspects of their interactions are minutely visualised. Accordingly the object in this work has been to determine, in as much detail as is possible, the life history of the individual cells in the several differentiating cell streams.

An account of some of this work (9–11), particularly where it relates to pigment formation, has already appeared. The present account will be divided for convenience into three Parts. *Part 1* will deal with the formation of the cortex and the structure of keratin, *Part 2* with the cuticle, and *Part 3* with the inner root sheath. The section describing materials and methods included here applies to all three parts. A description of the outer root sheath, and the associated dermal structures of the hair, including the papilla, will be given in subsequent papers.

Materials and Methods

Follicles from man, rat, mouse, and sheep have been studied, but this report will be confined to the human follicle on which most of the work has been done. In some animals portions of skin may be excised, fixed, and embedded. This procedure was not often practicable in the human case; but the simple operation of plucking was found to provide satisfactory material. The parts of the follicle which are removed by plucking vary from one individual to another and also with the particular phase of the hair growth cycle. For the limited purpose of studying the growth of the hair in the steady state, follicles with a well developed bulb attached were chosen. In most cases the plucked follicles were without papilla and germinal layers, but occasionally the complete papilla and its enclosed structures were obtained. Immediately after it had been plucked, the root was immersed in the standard buffered osmium tetroxide fixative (25) (1 per cent OsO₄ and veronal-acetate buffer at pH 7.3, cooled to about 5°C.). The follicles were fixed usually for 2 hours, after which they were washed, dehydrated, and embedded in the usual way. Mixtures of one part methyl and five parts butyl methacrylate were used for embedding. Some preparations were embedded in an epoxide resin.[1] A few special preparations of hair or chemically treated hair and wool will be described in the text.

The sections were cut with a modified Cambridge rocker microtome and examined with either a Siemens Elmiskop 1 or a Philips E.M.100 electron microscope.

Location of Micrographs

Longitudinal and cross-sections of the lower quarter of the follicle, which includes the whole of the region in which cell growth and differentiation take place, were obtained in the course of this study.

The location of the regions shown in the figures will be facilitated by reference to Text-fig. 1.1, which shows a *longitudinal section of the lower part of a follicle*. The follicle is shown divided into two symmetrical halves by a vertical line. On the left of this line are shown the several zones into which, for convenience in the subsequent discussion, the base of the follicle may be divided;

[1] Supplied by Aero Research, Ltd., Duxford, Cambridge, England.

THE ELECTRON MICROSCOPY OF THE HUMAN HAIR FOLLICLE*

PART 1. INTRODUCTION AND THE HAIR CORTEX

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PLATES 50 TO 53

INTRODUCTION

This series of papers will describe the fine structure of the hair follicle as revealed by electron microscopy of thin sections. Particular attention has been given in the study to the differentiation of the various cell layers and to the synthesis of the intracellular products.

There are many descriptions of the hair follicle in the literature, and the most useful have been found to be those by Hoepke (17), Maximow and Bloom (21), and Sharpey-Schafer (30) on human hair and Auber (6) on wool. The recent book by Montagna (24) contains a valuable review of the histochemistry.
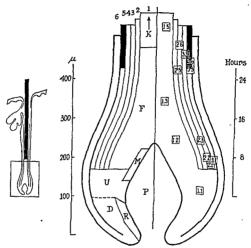
From the matrix of the follicle (Text-fig. 1.1) six distinct cell streams may arise, as a consequence of continuous cell division, to form the hair and the inner root sheath. Together these structures comprise· (1) the medulla, (2) the hair cortex, (3) the hair cuticle, (4) the cuticle of the inner root sheath, (5) the Huxley layer, and (6) the Henle layer. Layers 1, 2, and 3 comprise the emergent hair, layers 4, 5, and 6 the inner root sheath. The earlier work by light microscopy has shown that the differentiation and development in each of these cells are distinct from those in adjacent streams.

The hair follicle is an attractive object for a study of morphogenesis and differentiation for several reasons. First the hair is a small organ, not too large for surveying at high magnifications with the electron microscope; second there are available extensive chemical and histochemical data to be correlated with fine structure; finally the organ, since it is on the external surface of the

these contacts will be discussed in Parts 2 and 3. In contrast to their behaviour in the cuticle and sheath, the contacts in the presumptive cortical cells remain localised and intercellular gaps (up to 2 to 3 μ) are common. These gaps persist as the cells flow past the melanocyte rest (M) and leave the bulb. At this level (region F, Text-fig. 1.1) they rapidly elongate and the intracellular inclusions, nuclei, and mitochondria become oriented parallel to the axis of the follicle (Fig. 1.2).

TEXT-FIG 1.1 Key to location of electron micrographs The drawing shows a longitudinal section of the bulb region of a human hair follicle (shown in full in the inset). P is the papilla, R the germinal layer, D division region, U the undifferentiated matrix, M the zone of melanocytes. The regions of the hair and sheaths, indicated by the small numbers 1 to 6, are the cortex, hair cuticle, sheath cuticle, Huxley's layer, Henle's layer, and the outer root sheath respectively. The numbers enclosed in squares are those of the micrographs illustrating the text. The first number refers to the part and the second to the number of the figure, e g., 3.1 refers to Fig. 1 of Part 3. The position of the square indicates the region covered by the section. Certain figures, whose location is above the area shown on the diagram, are not indicated F is the region in which fibrous keratin is formed and K is the beginning of the keratinisation zone

the limits of these zones are only approximate. On the right of the line the locations of the various electron micrographs are indicated. The numbers in the squares are the numbers of the particular micrographs; e.g., 1.3 is Fig. 3 of Part 1. In most cases these locations were determined both by light microscopy of thick sections, cut adjacent to those examined in the electron microscope and also by low power electron micrographs. The time scale on the right hand side has been added to show the rate of movement of the cell stream and the duration of the various changes. It has been computed by assuming an average value of 0.3 mm./24 hours for the rate of growth of hair (29). Since no measurements were possible of the growth rate of the actual hairs used, the scale can only be approximate.

The Formation of the Cortex and the Structure of Keratin

OBSERVATIONS

The Undifferentiated Matrix.—All the cells forming the hair and the inner root sheath derive from the undifferentiated matrix (U in Text-fig. 1.1). Below this zone is the germinal layer R in which the cells have a columnar form and are attached at one end to the basement membrane of the papilla. A fuller account of the germinal layer will be given in a subsequent paper describing the papilla. The presumptive cortical cells are derived from the inner part of the undifferentiated region U (Text-fig. 1.1); they form the solid cylinder of cells, which in non-medullated hairs, occupies the central part of the follicle.

The nuclei of the cells of the matrix are roughly spherical, sometimes with a single indentation; they are extremely granular particularly those regions adjacent to the nuclear membrane. The nuclear/cytoplasmic ratio is large (Fig. 1.1). Nucleoli are conspicuous and granular. In the zone D typical division figures were occasionally noted. The mitochondria (M) are long and thin, but otherwise appear normal. There are regions containing clusters of empty agranular vesicles, which are similar to the Golgi regions described in other cells. The most characteristic feature of the cytoplasm is the numerous clusters of small dense particles (150 to 200 A diameter) (Fig. 1.1 P). The cell cytoplasm is relatively empty at this level and no accumulation of amorphous material, which might be a precursor of the fibrous keratin, has been observed.

The cells are rounded, but their surfaces are thrown into numerous corrugations and finger-like projections or pseudopods. Contact between the cells is limited to small local areas, which are made conspicuous both by the increased density of the membranes and also by the material which occupies the space between them (120 to 150 A). Contacts of a similar, but more elaborate kind, are very conspicuous in the outer root sheath and in the epidermis (28, 33). In the presumptive cells of the cuticle and sheaths these contacts spread and the surface irregularities are smoothed out. The morphogenetic importance of

Although sections of the fully hardened hair can be cut only with difficulty, the results have shown (Fig. 1.8) that the keratin has a rather uniform appearance in cross-section and the fibrillar structure is rarely visible. In unstained and unfixed material the cell membranes, nuclei, and other condensed inclusions, appear more dense than the keratin. When the keratin is removed, by first oxidising the cystine with peracetic acid and then extracting the oxidised protein with dilute alkali (1), these non-keratinous constituents are readily demonstrated (Fig. 1.9). The fibrillar structure in hair becomes more apparent after prolonged treatment of the hair in the osmium fixative, or after a preliminary reduction combined with heavy metal staining.

DISCUSSION

Differentiation in the Bulb.—A discussion of differentiation will be found in Parts 2 and 3 of this series following the description of the cuticle and inner root sheath. However, one aspect of the cell membranes may be conveniently discussed here. In the matrix (*M*, Text-fig. 1.1) the outlines of the cells are very irregular and intercellular gaps are common. These features may be interpreted as evidence of flexibility and lack of adhesiveness of the plasma membranes. In the presumptive cortex, in contradistinction to the cuticle and inner root sheath, this condition persists until the level of the neck of the follicle. The long melanocyte processes seem able to take advantage of the gaps to penetrate between the cells (10). Their tips, packed with pigment granules, have been found sometimes completely within cortical cells and sometimes partly enveloped by the cell membranes. It is possible that an active phagocytosis by the cortical cells is the mechanism by which the pigment enters the cells.

Protein Synthesis.—All the cells of the matrix have similar cytoplasmic constituents, although they subsequently make very different products. There are many mitochondria, a few agranular vesicles (Golgi vesicles ?), and many small dense particles 150 to 200 A, that may be identified with Palade's "small particulate component" (26). The cells are rich in ribonucleic acid (16, 24). There is no organised endoplasmic reticulum (27), but the particles associate in small clusters, which later become centres of a denser ground substance. A similar situation is found in skin (28, 32), and may be common to all epidermal cells. In the cortex, when keratin filaments appear, they bear no obvious spatial relation to the particles, suggesting that any precursor formed on the particles passes to the filaments in a form, which is either not preserved, or too small to see. These epidermal cells function for a short time only (48 hours) and accumulate their products without secreting them externally. This is quite a different situation to that found in secretory cells which continuously synthesise and secrete protein; these cells develop an elaborate reticulum.

In the mid- and upper part of the bulb (region F, Text-fig. 1.1) the gaps between the cortical cells are occupied by the long processes of the melanocytes. Pseudopods still project from the cortical cells at this level and seem active in the neighbourhood of the pigment-bearing ends of the processes. Occasional bundles of pigment granules, surrounded by two membranes, have been found within the cortical cells.

The Formation of Keratin.—Fibrous keratin first appears as wispy clumps of fine filaments in the cells of the mid- and upper bulb (F in Text-fig. 1.1). These filaments, less than 100 A in width, which seem to be definite structural units, are oriented parallel to the elongated nuclei, mitochondria, and the long axis of the cell. There is no obvious connection between the filaments and the clusters of dense particles. The particles seem to reside in patches of amorphous ground substance (Fig. 1.3).
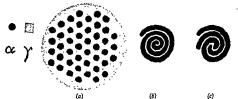
As the cells move towards the neck of the bulb, the bundles of filaments rapidly increase in length and width by the accretion of further filaments, until they reach a size visible in the light microscope. Histologists have previously referred to these fibrous structures as fibrils, therefore this term will be used here for such aggregates of filaments, but without restriction as to size. Many fibrils lie close to and parallel to the nuclear and cell membranes and some are twisted slightly around their long axis. The pigment granules lie in rows between the growing fibrils.

Keratinisation.—Above the constriction of the follicle is the region (K) (Text-fig. 1.1) referred to as the zone of keratinisation (6, 14). Here both the large fibrils and their component fine filaments become more clearly defined (Fig. 1.4). This is partly due to an increased regularity in the packing of the fine filaments, which over considerable areas approximates to hexagonal close packing, and partly to an increased contrast resulting from the appearance of a denser material between the filaments. The filaments within the fibrils now appear light on a darker ground (Fig. 1 6). Cells in which this change had occurred were found contiguous to others yet unchanged (Fig. 1 4). In addition to the hexagonal arrangement of the fine filaments, other patterns resembling those of finger prints are common. These patterns will be referred to as "whorls" (Fig. 1.7).

The large intercellular gaps, common at a lower level, disappear in the neighbourhood of the neck of the follicle. However, higher in the keratinisation zone, a uniform dilation of the membranes occurs, which results in a final spacing of about 200 to 250 A. A similar separation of cell membranes occurs in the cuticle and sheaths and will be described in Parts 2 and 3.

In the keratinisation zone more filaments together with interfilamentous material are added to the existing bundles of filaments, which become rather more uniform in diameter. Finally many fibrils fuse laterally to form large masses in the interstices of which the nuclear and cytoplasmic remnants are trapped (Fig. 1.5).

Keratin, using this name for the hardened fibrils which ultimately fill the cells of the cortex, would thus not be of uniform composition at the electron microscope level. In a sense it may be said to consist of two proteins in close association, one of which is fibrous and the other amorphous (Text-fig. 1.2). It is desirable in view of the importance of this concept to point to chemical evidence in its favour. Goddard and Michaelis (15) dissolved wool in alkaline thioglycolate solution and fractionated it into two proteins. Similarly Alexander and Earland (1) oxidised wool with peracetic acid to destroy the cystine cross-linkages and extracted the keratin with dilute ammonia. The residue (Fig. 1.8) consists of cell membranes, nuclei, and other cytoplasmic constituents (23).

TEXT-FIG. 1.2. (a) Proposed structure of the keratin fibrils showing the α-filaments embedded in a continuous, amorphous, interfilamentous cement (γ). The α-filaments are about 60 A in diameter and are considered to be responsible for the α-type x-ray pattern. Ideally the arrangement of the filaments is hexagonal, but more frequently the whorl structures (b) and (c) appear.

(b) and (c) Arrangements of filaments within the keratin fibrils giving the appearance of whorls or finger prints which are shown in Fig. 1.7. The association of the filaments may be stronger in one direction than in the other. The spirals, which may be single (b) or multiple (as in c), develop around areas of quasihexagonal packing, perhaps as a result of a dislocation The geometrical similarity to surface structures formed during crystal growth may be noted.

The extract may be fractionated into two main constituents, one a fibrous protein (α-keratose) which gives the same oriented x-ray pattern as hair, and the other a lower molecular weight, higher sulfur content protein (γ-keratose) incapable of being drawn into fibres. It seems likely that α-keratose is derived from the α-filaments and γ-keratose from the interfilamentous cement. Accordingly the designations α- and γ-keratin will be used to label the two constituents of the keratin complex (Text-fig. 1.2). Alexander and Hudson (1) report that the γ-keratose amounts to 30 to 40 per cent of the extractable keratin of wool. The approximate figure, derived from the areas of the two constituents in the cross-section of fibrils in hair, is nearer to 50 per cent. This may be regarded as good agreement, since the amount of γ-keratin to be expected from the hair (5 per cent sulfur) is greater than in wool (3.5 per cent sulfur).

Keratin Formation.—The formation of the fibrous keratin itself can be divided into two stages, first the formation of fine filaments and second the condensation of these into larger fibrils.

The filaments begin to appear in quantity in the upper bulb, where it has previously been shown (31) that the birefringence rises rapidly. An oriented α-type x-ray diffraction pattern can also be obtained from the follicle immediately above the constriction (22). There is little doubt therefore in identifying the filaments as the crystalline fibrous component of keratin. Porter (28) and Selby (32) have described the appearance of similar bundles of fine filaments in epidermal cells. They will be referred to as α-filaments, and it is considered that their internal structure is essentially unchanged in the final hair. Their diameter cannot be measured very accurately at this level, but it is substantially less than 100 A. At a higher level, where it is possible to make more accurate measurements, the diameter lies between 60 and 80 A. In longitudinal sections the filaments reveal a granular appearance, which may be due to a poorly resolved fine structure.

The filaments, when first formed, cluster in loosely knit bundles which may be tactoids. They are oriented parallel to the fibre axis as soon as they appear. This orientation, which is common to all components of the cortex, may be due to the narrowing of the follicle above the papilla tip, which constrains the cells to adopt a long spindle shape. The nuclei suffer the same deformation and the elongated mitochondria are also oriented.

The subsequent condensation of the filaments into more coherent large fibrils seems a different type of association which can develop suddenly cell by cell (Fig. 1.4). The most remarkable feature is that the filaments are now seen to be separated by a new substance which is denser than the filaments themselves. Because it improves the compactness of the bundles of filaments, it may be inferred that it functions as an adhesive cement. The appearance of this interfilamentous cement coincides with the rapid increase in the thiol (SH) content of the cortex (13, 16, 24). Bahr (7) has emphasised that there is a strong reaction between osmium tetroxide and cysteine or cystine. Further the length of the follicle which is stained a very dark brown by the fixative coincides with the keratinisation zone. Therefore as the osmium functions as a visible stain for thiol groups in this region, it may also function as an electron stain. Assuming that the high apparent density of the interfilamentous substance is due to its osmium content, it may be inferred that this substance has a higher cysteine content than the filaments.

The "whorl" structure shown in Fig. 1.7 and Text-fig. 2 (*b*) (10) becomes conspicuous as keratinisation increases, although areas of hexagonal packing persist in the centre of the fibrils. The interpretation of the whorls cannot be definitely established from our present data. However they may indicate a small twist in the bundle of filaments developing in the later stages of growth.

EXPLANATION OF PLATES

PLATE 50

The magnifications of the electron micrographs are indicated by a line with the length shown.

Many of the micrographs contain dense spherical particles, often aggregated, about 100 Å diameter These are particles of colloidal gold, which are added to facilitate focusing.

Fig. 1 1. An electron micrograph of cells of the undifferentiated matrix of the hair bulb (region 1', Text-fig. 1). These cells have a high nuclear/cytoplasmic ratio. The nuclei (N) and nucleoli (n) are conspicuous. The cytoplasm contains mitochondria (M), Golgi elements (G), and numerous dense particles (P). The wide intercellular gaps (possibly here enhanced by fixation), suggest that the cellular adhesion is poor. The surfaces have numerous finger-like pseudopods (S). × 16,000.

Fig. 1 2. A longitudinal section of the cortical cells in the mid-bulb region where keratin filaments are rapidly accumulating The cells are greatly elongated in the direction of the axis of the growing hair Intercellular gaps appear at G and cell contacts at c' The mitochondria (M) are elongated and oriented. Clusters of dense particles (P) fill the cytoplasm. Fibrils of keratin (F) are seen running parallel to the long axis of the cells × 24,000.

Fig. 1 3. Micrograph at higher magnification of a fibril (F), which consists of a loose association of filaments (f). × 83,000.

Relation to X-Ray Diffraction Data.—The wide-angle, fibre-type x-ray diffraction pattern (α-pattern) common to all mammalian keratins (2, 8, 20) must arise from the oriented α-filaments. The slight twist of the bundles of filaments, which is inferred from the existence of the whorl structure may contribute to the arcing of the diffraction spots. The γ-keratin presumably contributes only to the diffuse haloes, which are found in all the x-ray photographs (4, 5). That the sulfur is largely in the amorphous fraction is corroborated by the fact that reduction of cystine followed by substitution of the H of the thiol group by alkyl radicals, makes little change in the fibre-type pattern. Any attempt to relate the chemical composition to the crystalline structure of the α-filaments is difficult, as Astbury has pointed out (3), in the absence of determinations of the amino acid composition of the two separate fractions, α- and γ-keratin.

The small-angle x-ray diffraction pattern given by many keratins contains both lateral and longitudinal spacings (8, 20), indicating structures within the range of electron microscopy. The lateral spacings of the order of 70 to 80 A may well arise from scattering by the complex, "filaments plus cement." This complex consists of a system of rodlets (diameter *ca.* 60 A), in approximate hexagonal array with centres spaced 70 to 80 A apart, embedded in cement of different scattering properties. The longitudinal long spacings cannot be accounted for so readily. The filaments appear granular in longitudinal sections, which suggests a poorly resolved fine structure, but no regular spacing of the order expected (100 to 200 A) has been observed.

Comparison with Electron Microscopy of Fragments of Fibres.—The present conclusions concerning the structure of keratin are in good agreement with those drawn from an examination of fragments of fibres which have been disintegrated by chemical procedures designed to reverse the process of keratinisation (19). Farrant, Rees, and Mercer (12) reduced, ethylated, and digested wool with pepsin to free the fibrils and obtained suspensions of fine filaments about 100 A in diameter; these are probably the same structures as those now found in sections. Similar preparations have been obtained by Jeffrey, Sikorski, and Woods (18), who, however, report that their filaments are associated in sheets. There is, in many sections (Fig. 1.7) of the whorls, a suggestion that the filaments are attached to the adjacent unit more strongly in the tangential direction than in the radial one; certain forms of disintegration might separate these into sheets.

SUMMARY
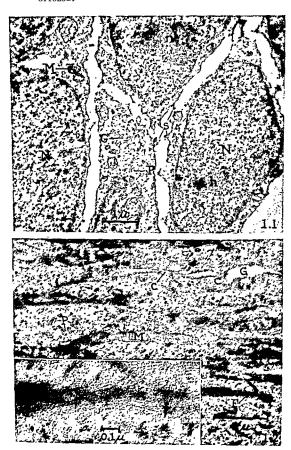
1. The presumptive cortical cells of hair in the undifferentiated matrix of the bulb contain mitochondria, agranular vesicles, and many small dense R.N.P. particles, but no keratin, pigment granules, or endoplasmic reticulum.

2. In the mid-bulb region intercellular adhesion is limited to small localised

areas. Intercellular gaps are common and the cell surfaces are irregularly convoluted. The melanocyte processes penetrate the cell gaps. The relation between their pigment-bearing tips and the involutions of the cell membranes suggests an active phagocytosis of the tips.

3. Fibrous keratin first appears in loose parallel strands of fine filaments (*ca.* 60 A diameter) in the mid-bulb. The filaments, the long mitochondria, and elongated nucleus are all parallel to the long axis of the cell and the axis of the follicle.

4. At the level of the constriction of the bulb and above, a dense amorphous substance appears between the fine filaments and apparently acts as adhesive cement. The bundles of filaments now form well defined fibrils. The packing of the filaments within the fibrils is in places hexagonal and elsewhere in the form of "whorls."

5. At higher levels further filaments and interfilamentous cement are added together with the whole cytoplasmic space becomes packed with fibrils which finally condense to massive blocks of keratin. The residual cellular material occupies the interstices.

6. The addition of the interfilamentous substance is regarded as an essential factor in keratinisation. Keratin is considered to be a complex made of fine filaments (α-filaments) embedded in an amorphous substance (γ-keratin) which has the higher cystine content.

7. The wide-angle fibre-type x-ray pattern is thought to be due to scattering by the fine α-filaments and some low angle lateral spacings to the filament-plus-cement structure.

REFERENCES

1 Alexander, P., and Hudson, R. F., Wool: Its Chemistry and Physics, London, Chapman and Hall, Ltd , 1954.

2. Astbury, W T., Croonian Lecture, *Proc. Roy Soc. London, Series B*, 1947, **134**, 303.

3. Astbury, W. T., *Proc. Internal. Wool Textile Conference*, Australia, 1955, in press.

4 Astbury, W. T., and Street, A., *Phil. Tr. Roy. Soc. London, Series A*, 1931, **230**, 75.

5. Astbury, W T., and Woods, H. J., *Phil Tr. Roy. Soc. London, Series A*, 1933, **232**, 333.

6. Auber, L., *Tr. Roy. Soc. Edinburgh*, 1952, **62**, 191.

7. Bahr, G. F., *Exp. Cell Research*, 1954, **7**, 457.

8. Barnicot, N A., Birbeck, M. S. C., and Cuckow, F. W., *Ann. Human Genetics*, 1955, **19**, 232.

9. Bear, R. S , and Rugo, H. J., *Ann New York Acad. Sc:*, 1951, **53**, 627.

10. Birbeck, M. S. C, and Mercer, E. H., *Proc. Internat. Wool Textile Conference*, Australia, 1955, Vol. F, 215.

11. Birbeck, M. S. C., Mercer, E. H., and Barnicot, N. A., *Exp. Cell Research*, 1956, **10**, 505.

(Birbeck and Mercer: Human hair follicle. Part I)

EXPLANATION OF PLATES

PLATE 50

The magnifications of the electron micrographs are indicated by a line with the length shown

Many of the micrographs contain dense spherical particles, often aggregated, about 200 A diameter. These are particles of colloidal gold, which are added to facilitate focusing.

FIG 1.1. An electron micrograph of cells of the undifferentiated matrix of the hair bulb (region U, Text-fig. 1). These cells have a high nuclear/cytoplasmic ratio The nuclei (N) and nucleoli (n) are conspicuous. The cytoplasm contains mitochondria (M), Golgi elements (G), and numerous dense particles (P). The wide intercellular gaps (possibly here enhanced by fixation), suggest that the cellular adhesion is poor. The surfaces have numerous finger-like pseudopods (S). $\times$ 16,000.

FIG 1.2. A longitudinal section of the cortical cells in the mid-bulb region where keratin filaments are rapidly accumulating. The cells are greatly elongated in the direction of the axis of the growing hair. Intercellular gaps appear at G and cell contacts at C. The mitochondria (M) are elongated and oriented. Clusters of dense particles (P) fill the cytoplasm. Fibrils of keratin (F) are seen running parallel to the long axis of the cells $\times$ 24,000.

Inset —Micrograph at higher magnification of a fibril (F), which consists of a loose association of filaments (f). $\times$ 80,000.

12. Farrant, J. L., Rees, A. L. G., and Mercer, E. H., *Nature*, 1947, **159**, 535.
13. Giroud, A., and Bulliard, H., *Arch. Morphol.*, 1930, **29**, 7.
14. Giroud, A., and Leblond, C. P., *Ann. New York Acad. Sc.*, 1951, **53**, 613.
15. Goddard, D.R. and Michaelis, L., *J. Biol. Chem.*, 1934, **106**, 605; 1935, **361**, 112.
16. Hardy, M. H., *Am. J. Anat.*, 1952, **90**, 285.
17. Hoepke, H., *Handb. mikr. Anat.*, 1926, **1**, 19.
18. Jeffrey, G. M., Sikorski, J., and Woods, H. J., *Proc. Internat. Wool Textile Conference*, Australia, 1955, Vol. F, 130.
19. Lindberg, J., Mercer, E. H., Philip, B., and Gralen, N., *Textile Research J.*, 1949, **19**, 673.
20. Macarthur, I., *Nature*, 1943, **152**, 38.
21. Maximow, A. A., and Bloom, W., Textbook of Histology, London, W. B. Saunders Co., 1948.
22. Mercer, E. H., *Biochim. et Biophysica Acta*, 1949, **3**, 161.
23. Mercer, E. H., *Textile Research J.*, 1953, **23**, 388.
24. Montagna, W., The Structure and Functions of Skin, New York, Academic Press, Inc., 1956.
25. Palade, G. E., *J. Exp. Med.*, 1952, **95**, 285.
26. Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 59.
27. Porter, K. R., *J. Exp. Med.*, 1953, **97**, 727.
28. Porter, K. R., *Anat. Rec.*, 1954, **118**, 433
29. Rothman, S., Physiology and Biochemistry of Skin, Chicago, University of Chicago Press, 1954.
30. Sharpey-Schafer, E., Essentials of Histology, London, Longmans, Green & Co., 1949.
31. Schmidt, W. J., Die Bausteine des Tierskorpers, Bonn, Fredrich Cohen, 1924.
32. Selby, C. C., *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 429.
33. Sjöstrand, F., Ottoson, D., Stenstrom, S., and Svaetichin, G., *Acta Physiol. Scand.*, 1953, **29**, 611.
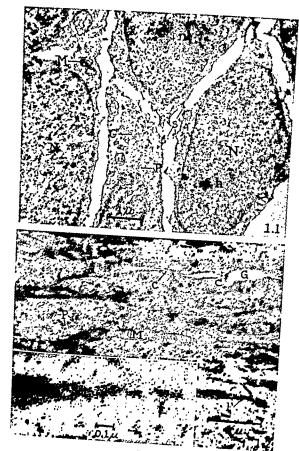
PLATE 51

Fig. 1.3. Cross-section of two cortical cells in the upper bulb region (about the same level as Fig. 1.2) showing cross-sections of fibrils at F. Cell membranes (B) showing gaps (G) cross the field. An elongated mitochondrion is cross-sectioned at M. The fibrils are loose and poorly consolidated in comparison with later forms (cf. Figs. 1.5 and 1.8). × 120,000.

Fig. 1.4. Cross-section of portions of two cortical cells immediately above the constriction of the bulb. Two adjacent cell membranes cross the field in a sinuous curve separating the two cells. These two cells show different degrees of keratinisation; the lower cell is at an earlier stage than the upper. The bundles of keratin filaments in the lower cell are less densely stained and relatively poorly organised. In the upper cell the bundles are more deeply stained (higher cysteine content) and a regular internal structure is developing, (quasihexagonal at H and in whorls at W). The bundles are more discrete and coherent and now may be recognised as fibrils. × 120,000.
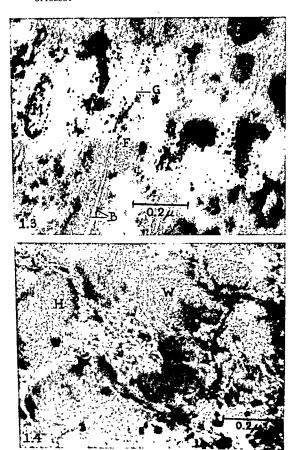
PLATE 52

FIG 1.5   Cross-section of cortical cells in the upper keratinous zone showing an advanced stage of fibril formation. Nuclei (*N*) are granular and condensed but their membranes can still be seen. Cell membranes appear at *B* The large keratin fibrils are shown in cross-section and almost fill the cytoplasm. Their diameter varies and some are fusing into massive formations which run parallel to nuclear and cell membranes, but from which they are separated by a distinct gap Other cytoplasmic components (particles *P* and mitochondria *M*) are crowded within the interfibrillar space. Sections of pigment granules are shown at *G*. × 24,000.

FIG. 1.6.   Micrograph at higher magnification of a cross-section of a cortical cell in the upper keratinous zone (same as Fig. 1.5) showing cross-sections of several massive keratin fibrils The fine filaments (α-keratin) appear light on the darker ground (γ-keratin). It is probable that the interfilamentous material is strongly stained by reaction with the osmium fixative, presumably due to its high cysteine(cystine) content. (See Text-fig 2)

The filaments within the fibrils are in some areas packed in a quasihexagonal array; in other areas the whorl formation may be seen (see also Fig. 1.7). Fusion of the fibrils into a larger continuous structure of rather uniform texture may also be seen The ill formed material between the fibrils is the cytoplasmic residue × 120,000
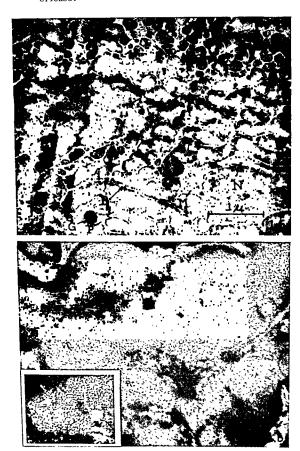
Inset shows a reversal of a portion of the section. Here the filaments appear as dark dots on a light ground × 120,000.
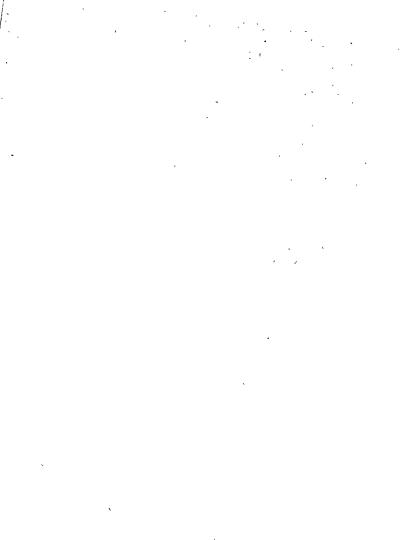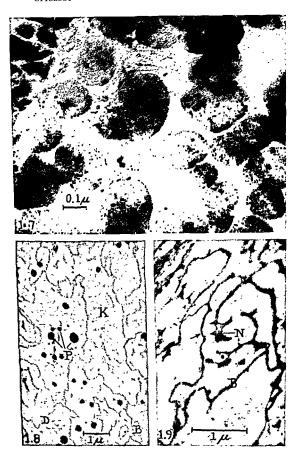
PLATE 53

Fig. 1.7. Cross-section of a part of a cortical cell in the upper keratinous zone with keratin fibrils showing whorl formations. These formations become more obvious as keratinisation proceeds, and may be due to the packing of the fine filaments which is a result of the cementing action of the dense interfilamentous cement (γ-keratin in Text-fig. 1.2). In some limited areas particularly in the centre of the fibrils the packing is hexagonal; peripherally the structure usually develops a whorl, characterised by the sections of the filaments (light areas) forming parallel lines separated by darker layers of cement × 80,000.

Fig. 1.8 Cross-section of the cortex of a fully hardened hair showing the homogeneity of the condensed keratin (K) Pigment granules (P), the interlocking cell membranes (B), which are denser than the keratin, and various dense cytoplasmic inclusions (D), may be seen. × 12,000.

Fig 1.9. Cross-section of the cortex at the same level as Fig. 1 8 after the extraction of the keratin (for method see text). The cell membranes remain as dense structures (B), nuclear and other remnants appear at N. The membranes appear as double lines at arrow and an intermembranal deposit is still present. × 25,000.
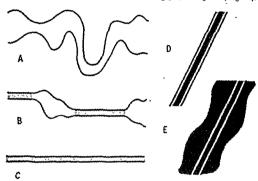
(Birbeck and Mercer: Human hair follicle. Part I)

(563)

(Birbeck and Mercer Human hair follicle Part I)

papilla height, between the lower and mid-bulb. This smoothing out is the result of small areas of contact being formed between successive cuticle cells (Fig. 2.1). In these areas, which are initially only about 1 micron in diameter, the two plasma membranes run strictly parallel about 100 to 150 A apart. The gap between them is filled with a material, which although less dense than the plasma membranes themselves, is significantly more electron-scattering than the material in the wider intercellular gaps (Text-fig. 2.1B, Fig. 2.2).

TEXT-FIG. 2.1. A diagrammatic representation of the various appearances of cuticle cell membranes. A represents the two membranes of two adjacent matrix cells. B indicates the first localised contacts, with cell cement between, and C the complete contact of two cells D shows the intercellular layer higher up the cuticle, and E the final appearance of the membranes in the emergent hair.

The plasma membrane itself increases either in density or in thickness in these areas. There is a similar observation by Porter (8) on the epidermal cells of Amblystoma larvae in which he described a thickening of the membrane. However the fibrous intracellular tufts observed by him were not seen in the hair cuticle.

These areas of contact rapidly increase both in number and in area, so that at a level of about half the height of the papilla, they have completely spread over the surface between the cuticle cells (Text-fig. 2.1C). Although the cells of the inner root sheath are also in close contact at this level, the cuticle cell membranes are denser and easily distinguished (Fig. 2.3). The cementing together of the surfaces of the cuticle converts the unorganised irregular cells into

THE ELECTRON MICROSCOPY OF THE HUMAN HAIR FOLLICLE*

PART 2. THE HAIR CUTICLE

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PLATES 54 to 56

(Received for publication, September 18, 1957)

INTRODUCTION

The cuticle of the hair is derived from the cells in the centre of the matrix of the hair follicle. As these matrix cells move upward, a special cylinder, one cell thick, differentiates and finally becomes the highly keratinised, scale-like cells of the emergent hair.

In an earlier work (2) using the sectioning method a few incidental observations on the cuticle were made. Except for this work the only other observations using the electron microscope have been obtained on wool either by the replica method (12) or by disintegration methods (5).

Details of the materials and methods which have been used in this investigation are described in the first paper of this series.

OBSERVATIONS

The morphology of the undifferentiated matrix cell has been described in the first paper of this series, and therefore the description of the cuticle will start with the first detectable change in this layer of cells. One observation on the plasma membrane of the matrix cell must, however, be described again, for it is this particular structure that is the first to be modified in what represents the first step in the differentiation of the cuticle. The membrane of the matrix cell appears in an electron micrograph as a thin black line which outlines the cell. This outline is extremely irregular; the cell surface is thrown into numerous pseudopods and invaginations (Text-fig. 2.1*A* and Fig. 2.1). It is the smoothing out of the membrane that is the first detectable sign of the differentiation of the cuticle. This occurs at about a third of the

plasm condenses and the small gap between the keratin and the outside membrane of the cell disappears. The resulting cell appears almost homogeneous (Fig. 2.7). The fine structure of the intercellular layer is also lost at this stage leaving only a broad band between the two cuticle cells (Text-fig. 2.1E). The cell now appears as a rigid dehydrated structure and it is presumed that no further morphological changes take place. In the condensed cytoplasm of the cell the nucleus remains as a flattened disc along with the remnants of mitochondria and other inclusions.

Some of the details of the final transformations of the cuticle cell are obscure owing to technical difficulties. The hardness of the fully keratinised structures makes sectioning difficult and the homogeneity of the condensed cell gives poor contrast. However, by examining the final hair after various chemical treatments, a few additional observations were made which confirm the structure of the cuticle cell.

When the residue resulting from prolonged digestion of hair by trypsin was sectioned, it was possible to identify cuticle remnants (Fig. 2.8). The layer corresponding to the condensed cytoplasm is removed, while the keratinised component remains intact. In a second experiment, hair was oxidised with peracetic acid and then extracted with ammonia to remove the keratin type protein (1). Sections of hair treated in this way (Fig. 2.9) show that the keratinised region of the cell has dissolved, but that the dense outer layer and the condensed cytoplasm remain. In a third experiment hair was fixed for an excessive time with osmium tetroxide, in an attempt to stain the cystine component of the cuticle cell. Sections of this hair (Fig. 2.10) again show that the keratinised region may be distinguished from the condensed cytoplasm and the resistant outer layer of the cell.

<div align="center">DISCUSSION</div>

Differentiation of the Cuticle.—In a differentiated tissue the plasma membranes are usually seen in sections as thin parallel lines separated by a rather constant distance of the order of 100 to 150 A. On the contrary in the undifferentiated matrix of the hair follicle, as pointed out in Part 1, the cell surfaces are wrinkled and intercellular gaps are common. Close contact, with a spacing of the order of 100 to 150 A, is found only over very limited areas. This situation suggests that the membranes of undifferentiated cells are not very sticky. In the presumptive cuticle immediately above the matrix, the areas of close contact increase and, as a consequence, the gaps disappear. This development occurs a little later in the inner root sheath. In the presumptive cortex, on the other hand, the gaps do not disappear until the cells reach the neck of the bulb (Text-fig. 1.1 in Part 1). The existence or absence of intercellular adhesion thus seems to be an early manifestation of differentiation.

The spread of the area of contact between cells seems to be due to the surfaces becoming more sticky and the substance responsible for this change could

an organised layer of cuboidal cells. It is a rapid process and is completed in
the mid-bulb region of the hair follicle at the melanocyte level (M in Text-fig.
1.1, Part 1). As mentioned in Part 1, the cortical cells become pigmented at this
level—the cuticle is never pigmented.

The next change which the cuticle cell undergoes is primarily one of shape; it
is a slow change which is not complete until above the level of follicle constric-
tion. The cuboidal cells, already described, are sheared into the imbricate
condition seen in the final cuticle. During this process there are a few intra-
cellular modifications but no sign of keratin synthesis. A polarisation develops
which is shown by the nucleus moving toward the cortical side of the cell
(Fig. 2.3) and by the Golgi complex moving to the perinuclear zone away from
the cortex. As the cell tilts the nucleus moves to the lower end of the cell and is
squashed into a disc-like shape; but its internal structure is unchanged.

The other modification observed in the cuticle cell during this stage of its
development is the production of an amorphous or perhaps a very fine granular
(<50 A) material, which lines the inner side of the plasma membrane to a depth
of several hundred angstroms (Fig. 2.4). The plasma membranes themselves
remain as single dense lines about 150 A apart.

The cuticle cells reach their final imbricate condition at a level about two to
three times the height of the papilla or 400–500 μ from the germinal layer. At
this point there occurs simultaneously the formation of a number of inter-
cellular sheets and the first appearance of keratin. It was observed that, in
several hairs, this particular level of the cuticle coincided with the level at
which the trichohyaline of the Henle cells becomes fibrous (see Part 3). The
two plasma membranes separate to about twice their original width and sheets
of dense material appear in the enlarged intercellular spaces. These sheets con-
sist of two thin very dense layers separated by a central, thicker, less dense
layer which is sometimes divided by another thin, dense layer. The whole com-
plex is separated from the adjacent plasma membranes by narrow light spaces
(Figs. 2.5 and 2.6, Text-fig. 2.1D).

An intercellular complex occurs also between the inner root sheath cells (see
Part 3) and therefore its significance will be discussed in the third paper of this
series.

The keratin of the cuticle first appears as small amorphous droplets about
300 A in diameter (Fig. 2.5) They move towards the sheath side of the cuticle
cell, and coalesce in larger lumps which remain separated from the plasma mem-
brane by a narrow space. The keratin, as it collects, tends to form "bars" with
small gaps which run toward the cortical side of the cell (Fig. 2.6).

The deposition of keratin continues for several hundred angstroms until the
bars of keratin extend inwards about two-thirds of the cell width (Fig. 2.6). The
spaces between the bars are then filled to form a solid mass of keratin occupying
two-thirds of the cell. Simultaneously with this last stage, the remaining cyto-

hair with the light microscope, have attempted to explain the upward tilt of the distal ends of the cuticle cells by supposing that the inner root sheath moves more rapidly than the cortex. There is no convincing electron microscopic evidence for or against this hypothesis. However, since the flattening of the cuticle cells could be explained by Schmitt's hypothesis of a zipper-like spread of cell contacts, it may be possible to extend the theory to account for the tilt of the cells. It would be necessary to suppose that the cuticle cells stick more strongly to the inner root sheath than to the cortex and creep upwards at their distal tips. This would provide a bias to ensure that the cells would tilt in the observed direction as the cell formation narrows at the neck of the follicle.

Keratinisation of the Cuticle.—The cortical cell starts making fibrous keratin *at the mid-bulb level, while the production of the amorphous keratin in the cuticle starts a distance of several hundred micra later.* It is known that the cuticle keratin differs from the cortical fibrous keratin in having a higher sulfur content (3). Its high electron density would also suggest a high concentration of —SH; it therefore might be similar to the high sulfur component of the cortex (γ-fraction), which is amorphous, and also appears at about this level. (See Part 1.) The residue of the cytoplasm, which occupies the inner region of the flattened cell (Fig. 2.6), *also undergoes a chemical change and becomes insoluble in keratinolytic solvents.*

The distribution of keratin in the cuticle, filling only the outer region of the cell, demonstrates the radial polarisation of the cuticle, which was also shown, by the position of the nucleus and the Golgi complex, at an earlier phase of the cells' development. In this respect the cuticle is unique, since all the other cell layers of the hair have an axial orientation. This layered structure of the cuticle agrees with that *earlier inferred from electron microscope studies on fragmented* wool cuticle cells (5).

In addition to the keratinised region and the condensed cytoplasmic region of the cuticle cell, there is also a thin outermost layer which is particularly resistant to both trypsin digestion and keratinolytic attack (Figs. 2.8 and 2.9). This layer may be derived either from the extracellular sheets or from the small gap between the plasma membrane of the cell and the keratin layer, which may fill with a resistant material. Unfortunately, not only is the derivation of this layer unknown, but also the nature of the layer on the outermost cuticle cell is uncertain. The membrane complex that forms between the hair cuticle and the cuticle of inner root sheath, is somewhat different from the intercuticular sheets; but the fate of this membrane, when the inner root sheath breaks down, was not observed.

. This resistant layer on the surface of the cuticle may be related to the existence of a thin resistant membrane called the "epicuticle." This structure has been postulated to explain the production of bubbles and continuous tubules, which can be separated from the hair by various chemical treatments (5). If

be the material of low "electron density," which is observed between the plasma membranes when these are in close contact (Text-fig. 2.1B, Fig. 2.2). The early formation of this adhesive, intercellular substance could thus be a crucial step in the differentiation of the cuticle.

The increased adhesion and the increase in density of the plasma membranes themselves may be factors controlling other aspects of the development of the cuticular layer. Schmitt (10) has shown how a zipper-like spread of the area of contact can convert a layer of cells into a columnar formation. The observed changes in the cuticle seem very like an illustration of this suggestion. Further, the dense, well cemented membranes could easily be supposed to deprive the cells of food and account for their slower rate of synthesis

In Part 1, it has been suggested that the cell membranes of the cortical cells actively participate in the acquisition of pigment by the phagocytosis of the melanocyte tips. Such an activity would be impossible were the cell membranes firmly cemented together. Since this is the case at the level where the melanocyte processes reach the presumptive cuticle, it may account for the absence of pigment in this layer and in the inner root sheath.

Cell Membranes.—Electron micrographs of most cells show that the cytoplasm is bounded externally by a thin, dense membrane which may be called the plasma membrane. Although this membrane is usually less than 30 A thick, it can be thickened and increased in density locally to form the nodes, described in epidermal cells by Porter (8), Selby (11), and Sjöstrand (13), and observed, in the present work, in the outer root sheath of the hair follicle.

Whether the other layers, often associated with the plasma membrane, are to be regarded as part of the cell membrane is probably largely a matter of definition. When cells are in close contact in a stabilised tissue, the intercellular space (about 100 to 150 A wide) is occupied by a material of rather low electron density. This material, which appears to function as a cement, might be regarded as part of the "cell membrane complex." Robertson (9) for instance, arguing from his observations on the myelin sheath, has suggested that this surface layer, which he supposes is composed of several lipide layers, is an integral part of the cell. However, the observations on epidermal cells show that neither the plasma membrane nor the external layer is constant in morphology. Moreover the external layer seems to be present only between cells in contact and for this reason it may be best regarded as an extracellular secretion.

A third component which is associated with the plasma membrane, is the amorphous layer coating the cytoplasmic side of the cuticle membrane, particularly at the level where the cells tilt. This layer may be a precursor of the extracellular cement, but is probably not an essential part of the plasma membrane. However Mitchison (6), in his membrane model, has postulated a loosely packed layer of protein in this intracellular region, the function of which is mainly that of a mechanical support.

The Imbricate Condition of the Cuticle.—Authors (4, 7), who have examined

hair with the light microscope, have attempted to explain the upward tilt of the distal ends of the cuticle cells by supposing that the inner root sheath moves more rapidly than the cortex. There is no convincing electron microscopic evidence for or against this hypothesis. However, since the flattening of the cuticle cells could be explained by Schmitt's hypothesis of a zipper-like spread of cell contacts, it may be possible to extend the theory to account for the tilt of the cells. It would be necessary to suppose that the cuticle cells stick more strongly to the inner root sheath than to the cortex and creep upwards at their distal tips. This would provide a bias to ensure that the cells would tilt in the observed direction as the cell formation narrows at the neck of the follicle.

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the resistant layer, which has been described above, is part of the extracellular complex membrane, then it will form a continuous extracellular sheath to the cuticle cells. Even if the resistant layer is formed intracellularly, it may be that a relatively poor mechanical adhesion between the resistant layer and the keratin layer of the cuticle, would allow this layer to be lifted as a continuous layer from the hair fibre.

SUMMARY

1. During the early differentiation of the cuticle the cell membranes smooth out and the cells become closely attached over most of their surface. The change seems to be due to a layer of cement which forms between them. The plasma membranes also increase in density.

2. The decreased membrane activity of the cuticle cells may prevent a phagocytosis of the melanocyte processes and thus account for the non-pigmentation of the cuticle.

3. The flattening and imbrication of the cuticle may possibly be explained by a zipper-like spread of cell contacts.

4. Keratinisation of the cuticle occurs at a late stage in its development; the keratin formed is an amorphous type, similar to the γ-fraction of the cortex which is produced at a similar level.

5. Keratinisation is accompanied by the formation of complex intercellular layers similar to structures observed in the inner root sheath (see Part 3).

6. In the final stage of keratinisation the remaining cytoplasm condenses with the result that the cell is divided into a laminated structure with an outer keratinised layer and an inner layer, which is insoluble in keratinolytic solvents.

REFERENCES

1. Alexander, P., and Hudson, R. F., Wool: Its Chemistry and Physics, London, Chapman & Hall, Ltd., 1954.
2. Barnicot, N. A., Birbeck, M. S C., and Cuckow, F. W., Ann. Human Genetics, 1955, 19, 232.
3. Geiger, W. B., Textile Research J., 1944, 14, 82.
4. Hoepke, M., Handb. mikr. Anat., 1926, 1, 19.
5. Lindberg, J., Mercer, E H , Philip, B., and Gralen, N., Textile Research J., 1949, 19, 673.
6. Mitchison, J. M., Symp. Soc. Exp. Biol., 1952, 6, 105.
7. Montagna, W., The Structure and Function of Skin, New York, Academic Press, Inc., 1956.
8. Porter, K., Anat. Rec , 1954, 118, 433.
9. Robertson, J. D., J. Biophysic. and Biochem Cytol., 1955, 1, 271.
10. Schmitt, F. O., Growth, 1941, 5, suppl., 1.
11. Selby, C. C., J. Biophysic. and Biochem. Cytol., 1955, 1, 429
12. Sikorski, J., Proc. Internat. Wool Conference, Australia, 1955, in press.
13. Sjöstrand, F., Acta Physiol. Scand., 1953, 29, 611.
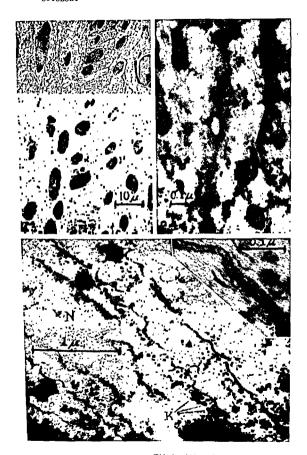
EXPLANATION OF PLATES

PLATE 54

Fig. 2.1. A low power electron micrograph of a longitudinal section from the mid-bulb region of the hair follicle. The outer root sheath is toward the top left and the papilla toward the bottom right; the general movement of the cells of the follicle is toward the top right. The cell (A) is the first cell which it is possible to identify as a cuticle cell; in the original section it was possible to trace the cuticle downwards via the cell (B) to the cell (A) as a continuous chain of closely adhering cells. The future of the remaining cells in the micrograph cannot be determined, although presumably the cells D, E, F, and G will become the inner root sheath, cells H and I the cortex, while the cell C will possibly become a cuticle cell. It may be observed that there are only a few local cell contacts between the cells at this level, with the exception of the contact between the cells A and B. Between the cells of the cortex there are large extracellular spaces through which melanocyte processes may be thrust; one such process (m) is seen (bottom right) although this represents the very lowest level of the follicle at which these may be found. $\times$ 6,000.

Fig. 2.2 An electron micrograph of a longitudinal section in the upper bulb region of the hair follicle. The membranes between two cuticle cells run from bottom left to top right. The two membranes are in good contact along most of their length and appear as a wavy line with only small areas not cemented together. Parts of the nuclei (N) may be observed top left and bottom right; the nucleus on the left has a typical invagination (i) of the nuclear membrane. Numerous mitochondria together with a fine granular component fill the cytoplasm The right hand cell has a well defined Golgi region (G). $\times$ 32,000.

Inset is a high power micrograph of a region of the two cell membranes at a point where they open up to form an extracellular space. The two parallel membranes in close contact may be seen on the right. The space between them is filled with a layer of cement C The layer of cement may also be observed lining the membrane around the extracellular space. $\times$ 160,000.

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(577)

PLATE 55
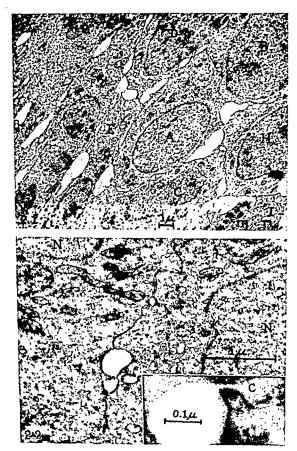
FIG. 2.3. A low power electron micrograph of a longitudinal section through the upper bulb region of the hair follicle. The cuticle is the vertical row of cells in the centre; on the left is the cortex, and on the right the row of small cells is the cuticle of the inner root sheath and beyond this, only partly in view, the Huxley layer. The cuticle cells at the bottom of the micrograph are cuboidal but towards the top they become slightly thinner and longer and somewhat imbricated. The membranes of the cuticle cells stand out much more obviously than the membranes of the cells in the other layers. The nuclei of the cuticle are located towards the cortical side of the cells. The spindle-shaped cortical cells are already synthesising keratin and there are droplets of trichohyaline in the Huxley layer. × 1,200

FIG. 2.4. An electron micrograph of the cuticle at the same level as on FIG. 2.3. The two plasma membranes of two cuticle cells run vertically up the centre of the micrograph. The two membranes run parallel with the exception of a small vesicle in the centre, and are cemented together with a low electron scattering material At this level the two membranes, beside being cemented, are straight (cf. Fig 2.2). Lining the two membranes is the intracellular layer of amorphous material. Both embedded in this material and also free in the cytoplasm are numerous "Palade" particles. In the cell on the right, part of the nucleus (N) may be observed. In both cells there are a few mitochondria × 60,000

FIG. 2.5. An electron micrograph of a cross-section through the cuticle at a level where the cells are beginning to synthesise keratin In the bottom left corner part of the cortex may be seen. The innermost cuticle cell contains a nucleus (N); at this level where the cells are extremely imbricated the nucleus is always at the lower end of the cuticle cell and therefore, in cross-section, appears in the innermost cell. The keratin (K) may be observed as small rounded dense drops which are forming near the sheath side of the cuticle cell; also in the cytoplasm there are numerous granules and a few mitochondria. × 40,000.

Inset is a high power micrograph of the membranes between two cuticle cells at a point where the complex intercellular layer is being formed. At the upper left are the two normal plasma membranes which may be observed to separate and between them (at the bottom right) the complex intercellular layer is present. The parallel intracellular membranes, which may be observed at the point where the layer is formed; we e only occasionally observed × 200,000

(Birbeck and Mercer Human hair follicle Part 2)

PLATE 56

FIG 2 6. An electron micrograph of a cross-section of the cuticle at a level where the cell is partly keratinised. Three layers of cuticle cells may be seen and beyond them, at the top right, the cuticle of the inner root sheath and in the extreme corner the Huxley layer. The bars of keratin may be seen on the sheath side of the cuticle cell with a small gap between them and the plasma membrane. The remaining cytoplasm is extremely granular. The complex layer may be observed between the cuticle cells ✕ 40,000.
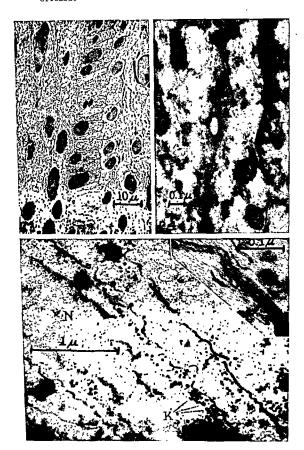
Inset is a high power micrograph of the complex layer. The small gap between the membrane and the developing mass of keratin may be observed. ✕ 200,000.

FIG 2 7. A high power electron micrograph of a cross-section of the completely keratinised cuticle cells The cells appear almost homogeneous; the keratin fills the sheath side of the cell, and the remaining cytoplasm, towards the cortex on the bottom left, is condensed. The two regions may be identified since the condensed cytoplasmic region is not quite so dense, and more granular than, the keratin. The central cuticle cell contains part of the pyknotic nucleus which has been forced toward the cortical side of the cell by the developing keratin The complex intercellular layer has also lost its fine structure and become uniformly dense. ✕ 120,000.

FIG 2 8 An electron micrograph of a cross-section of a single cuticle cell isolated from wool by tryptic digestion (7 days at pH 8 6 at 40°C.) The presumed position of the inner membrane of the cell is shown by a dotted line; the condensed cytoplasmic region of the cell. which lies between this line and the remaining part of the cell, has been extracted by the trypsin The keratinised region is unattacked; the thin dense layer above the keratin, which is also resistant, may be observed. ✕ 24,000.
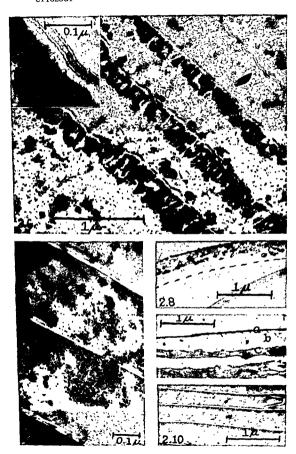
FIG 2 9 An electron micrograph of a cross-section of a wool fibre that has been oxidised with peracetic acid and extracted with ammonia in order to remove the keratin of the cell The micrograph shows that the leading edge of the cell appears as a thin dense line (a) which is resistant The keratinised region (b) is extracted, while the condensed cytoplasmic region of the cell (c) remains unattacked. ✕ 24,000

FIG 2 10 An electron micrograph of a cross-section of the hair cuticle after prolonged fixation (48 hours at room temperature) with osmium tetroxide. The resistant leading edge of the cuticle is selectively stained by this procedure (cf. Fig 2.7). There is also a slight differential staining of the keratinised region relative to the cytoplasmic region ✕ 24,000.

(Birbeck and Mercer: Human hair follicle. Part 2)

(577)

unite the cell membranes of neighbouring cells, appears to be the means by which permanent intercellular relations are established. However, neither do the plasma membranes become as dense as in the cuticular layer, nor does the spread of the contacts progress as far as the corresponding development in the cuticle. The cells of the presumptive sheath cuticle, which are smaller than the cells of the other layer, become closely united to the hair cuticle cells and are deformed in a similar way at a higher level.

Trichohyaline droplets appear in the cells of Henle's layer immediately above the undifferentiated matrix (Fig. 3.1). They are dense, rounded, structureless bodies, apparently without an enclosing membrane, which coalesce readily to form larger droplets. Similar droplets also appear in the cells of Huxley (Fig. 3.2) and sheath cuticle cells, but the rate of accumulation is greatest in Henle's layer, which is also the first to become fibrous.

The transformation of the amorphous droplets into the birefringent modification follows the same course in each of the three layers of the sheath. In Henle's layer at the level of the constriction of the follicle, the change occurs with dramatic suddenness. In the light microscope the disappearance of the large droplets and the conversion of the cells into a clear condensed birefringent layer can be seen (Figs. 3.3 a and b). Electron micrographs of precisely the same field (Fig. 3.3 c) show the same events in greater detail.

At a higher magnification in longitudinal sections of these regions "filaments" can be seen extending in both directions parallel to the follicle axis from lenticular shaped droplets of the amorphous form (Fig. 3.4 and 3.5 a). In cross-sections of the same levels the two forms can also be distinguished; the unconverted material appears structureless except that at the edges it may be fringed with ill defined filaments. The interpretation of the images of cross-sections of the converted form (Fig. 3.5 b) is uncertain. In some areas the sections show small dots (diameter ∼ 100 A) which are presumably sections of filaments; whereas in others the sections show wavy lines implying that the true form is a wavy sheet or tube. In the condensed form longitudinal sections show a dense array of parallel lines spaced somewhat less than 100 A apart (Fig. 3.5 a), together with small amounts of granular material which may be unconverted trichohyaline.

In the cells of the Henle layer the transformation proceeds rapidly to completion; the hardened coherent cylinder which results appears to act as a retaining mould for the softer tissues within it. The transformation occurs later (mid-keratinous zone) and proceeds more slowly in the cells of the Huxley and sheath cuticle layers, so that the two forms of trichohyaline coexist in the same cells over a considerable length of the follicle.

In the upper keratinous zone, where the three layers fuse, the cell contents present a dense compact appearance (Fig. 3.6); the boundaries of the cells are still clearly visible (see next section) and the nuclei are shrunken, elongated,

THE ELECTRON MICROSCOPY OF THE HUMAN HAIR FOLLICLE*

PART 3. THE INNER ROOT SHEATH AND TRICHOHYALINE

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PLATES 57 to 60

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INTRODUCTION

The cells of the three layers, Henle, Huxley, and the sheath cuticle, which together form the inner root sheath, arise from the peripheral portions of the undifferentiated matrix (see Text-fig. 1.1, Part 1). They form three concentric cylinders each one cell thick. Each layer has its own peculiarities, but it is convenient to consider them together because the type of intracellular product is the same in each and also, farther along the follicle, they form a single sheath, which later disintegrates while the hair itself persists.

The characteristic intracellular product is *trichohyaline*, a poorly characterised substance, presumably protein in nature, closely related to the keratohyaline of the epidermis. These substances are considered by many (see reviews in references 2 and 3) to be precursors of fibrous keratin. It will be shown that trichohyaline is certainly a precursor of the fibrous component of the sheath. However, the fibrous keratin of the cortex (see Part 1) does not seem to have a precursor of this type and is morphologically very different.

In this part the formation and transformation of trichohyaline and the development of the complex surface structures, which accompany the condensation of the sheath cells, will be described and discussed. The details of the methods used have been given in Part 1.

OBSERVATIONS

The early differentiation of the sheath cells may be followed in micrographs of the mid-bulb and upper bulb (Fig. 3.1). The course of events is similar to that already described for the hair cuticle. The spread of localised adhesions, which

all differentiating systems of cells pass through such stages. Indeed Abercrombie and Heaysman (1) have postulated "adhesive contact" and "surface inhibition" to explain the behaviour of cells in cultures seen in the light microscope. It may be valuable, therefore, now that all the growing layers of the follicle have been described, to summarise the development of the hair in more general terms.

Ideally the analysis of hair growth should commence with the formation of a new hair, but we have insufficient new information to attempt this and shall only, in a very tentative way, consider the conditions prevailing in a state of steady growth. In this state the presence of the previously differentiated cells above the matrix may provide a guiding pattern, or template, which would be absent in an entirely new growth.

In the bulb of the follicle we observe, dipping down into the cell matrix, a cylinder of altered cells, the lower end of the presumptive cuticle and inner sheath, which is continually able to recruit further cell members from the reservoir beneath. The cells at the end of this cylinder may attract their like from the matrix, or they may, by direct contact, induce changes in the cells immediately beneath them, which increases their mutual adhesion and thus extends the length of the existing formation. The first supposition implies either the pre-existence of differentiated cells or of some other unidentified factor, such as distance from the papilla or dermal layers. The latter supposition is at least in accord with the present observation. Since we observe that the spread of adhesive contact between cells precedes intracellular differentiation, it may be that, not only are the cells drawn together, but that these changes also influence the synthetic activities of the cells.

Contact induction of this kind has been postulated in embryology (see the review by Waddington (5) already referred to in Part 1) and Weiss (6) has made various suggestions as to how it can happen in molecular terms. In the present context it may be assumed that contact with the altered (sticky) cells of the cuticle and sheath biases a cell's synthetic apparatus towards the production of an adhesive cement. Thus the matrix cell is modified and added to the existing formation. The cuticle leads the way in this development and is closely followed by the sheath cells.

Thereafter the cells of the follicle are separated into two compartments, or special environments, in which the state of the cell membranes differs. The first is the cortical region, adjacent to the papilla and inside the cuticular "screen," in which fibrous keratin is the characteristic product. The second is the region, between the cuticle and outer root sheath, in which trichohyaline formation prevails. In the cortex (cf. Part 1) cell contacts remain local, gaps persist, and are exploited by melanocytes and the cell population is homogeneous. In this instance the failure to form cement seems to be the controlling factor. In the second region (the inner sheath) the cells closely adhere and

and denser than the transformed trichohyaline. The latter presents a rather uniform, fine texture of parallel lines in longitudinal section and close but randomly packed lines or points in cross-section (Fig. 3.6).

The Cell Membrane Complex

In the upper bulb region, where the relationships between the various cells of the hair and sheath are established, the membranes of the sheath cells become extensively attached over most of their surfaces as a result of the spread of the adhesive contacts discussed in Part 2. The intercellular attachments, at least in the layers of Huxley and Henle, are less complete than in the hair cuticle. That some degree of membrane mobility remains is shown, particularly in cross-sections, by surface convolutions. The two plasma membranes are spaced about 120 to 150 A apart and the intervening space is filled with the material of poor scattering power which, in Part 2, was identified as an intercellular cement. However, at the level of the fibrous transformation of trichohyaline, there occur striking changes at the cell surfaces which lead to the formation of thick complex intercellular layers.

The first sign of impending changes is the gathering beneath the plasma membranes of a dense material which is more abundant than the similar formations noted in the cuticle cells of the mid-bulb (Part 2). The plasma membranes then separate to a distance, which may be as great as 300 to 400 A (Fig. 3.7), and between them a dense deposit appears. At first ill defined and only locally distributed, this deposit eventually forms a broad, dense, central band in the midst of less dense material (Fig. 3.8). The "surface complex" uniting the two cells at this level thus consists of the two plasma membranes (spacing ~300 A), an intercellular "cement" of low scattering power, and the dense central band (width ~100 A). As the cells of Henle harden, owing to the condensation of the trichohyaline, this five banded formation persists and can be seen both in longitudinal and in cross-section. The cells of Henle are seen in longitudinal sections to be particularly closely interlocked by deeply indented surfaces (Fig. 3.3 c).

Very similar surface membrane complexes form between the other cells of the inner sheath (Fig. 3 6), but in the cuticle of the sheath the central band may be missing.

DISCUSSION

Early Differentiation.—The differentiation of the cells of the inner root sheath follows the pattern already described in Part 2. The structural change, which seems to initiate differentiation, is the formation and spread of areas of contact between the cells. This increased area of contact appears to suppress the surface activity of the cells and to stabilise their geometrical relationships. It seems unlikely that these phenomena are peculiar to the hair root; probably

The close histochemical similarities between trichohyaline and keratohyaline of the epidermis, suggest that a similar transformation occurs in the epidermis where it may account for the glassy appearance of the stratum lucidum.

Intercellular Deposits and Membrane Complexes.—The complex intercellular deposits, which appear between the sheath cells at the time of the transformation of trichohyaline, are similar in character to those occurring between the hair cuticle cells described in Part 2.

The material pervading the intercellular space seems to be a form of the intercellular cement which may be seen earlier between the cells of the upper bulb. Its composition is unknown. The lack of reaction with osmium tetroxide excludes many classes of compounds, but would not exclude polysaccharides. The rather definite spacing of the membranes in the mid-bulb and upper bulb (100 to 150 A) is similar to that commonly noted in other tissues. However, the surface complex in the sheath has a total width of as much as 300 to 400 A, with local irregularities reaching 500 A. It is difficult to see in these large and variable dimensions evidence of special structures, *e.g.* multilayers of lipides, protein films, etc., postulated to explain the dimensions of plasma membranes ~30 A thick. The dense bands could be sites of reactions between the cement and denser substances, *e.g.* calcium, distributed at first in a quasiperiodic manner but later more uniformly.

The function of the thickened membranes is also obscure. It may be significant that their appearance in time coincides with the intracellular changes leading to a hardening of the cell contents. This suggests that they may be necessary to hold the harder tissue together. An objection to this idea is that the Henle–outer root sheath complex is of the same "three line type" as elsewhere in the sheaths, whereas it is widely supposed that the Henle layer slides over the surface of the outer root sheath (see review in references 2 and 3).

SUMMARY

1. The three cylinders of cells, each one cell thick, which together constitute the inner root sheath, arise from the peripheral portions of the undifferentiated matrix. These cells, like the hair cuticle, are stabilised by the spread of adhesive contacts between their plasma membranes which occurs in the mid-bulb and upper bulb of the hair follicle.

2. The characteristic intracellular product of all three cell layers is trichohyaline. This substance is formed in the first place as amorphous droplets which subsequently transform into a birefringent form.

3. This transformation, involving the formation of a birefringent product from an amorphous precursor, is in contrast to the formation in the cortex of keratin which originates in a fibrous form.

4. Trichohyaline appears first and transforms first in the cells of Henle which are nearest the outer sheath and the dermal supply vessels. This trans-

contact induction could be the important factor. In this cylinder of cells, with the cuticle on one side and the outer sheath on the other, a structural gradient exists in a radial direction. Since the rate of trichohyaline synthesis declines as the distance from the outer sheath increases, it seems that the stimulus (and supplies) come from the outer sheath or the dermal surroundings.

The cuticular layer separating the two zones is anomalous. Synthesis is delayed and the product ultimately formed is a peculiar amorphous keratin of high cystine content. Again the influence of the cell membranes may be suspected, since these are more dense and the "zipper action" has drawn the formation into close internal contact.

Trichohyaline Formation.—Trichohyaline is as characteristic of the sheath cells as keratin is of the hair and thus represents an important expression of the synthetic potentialities of epidermal cells. Almost all the data relating to it, and to the keratohyaline of the skin, concern its staining properties in histological preparations from which it is not easy to determine its composition. It is assumed to be a protein, but whether it is of the α-type, or stabilised by cystine cross-linkages (3) is not known. It seems to be synthesised as a viscous insoluble liquid, not unlike the keratin of the hair cuticle. It is not accumulated in vesicles, nor is there any obvious spatial connection with the numerous clusters of dense particles.

The transformation of the "liquid" trichohyaline into its birefringent modification is unlike any intracellular phenomenon previously described. It resembles in many respects the crystallisation of a substance from the liquid phase. The remarkably perfect orientation of the birefringent form may be due, as in the cortex, to the slight flow which orients the cytoplasmic contents parallel to the axis of the follicle. The droplets of trichohyaline become lenticular in shape and the modified form extends axially from each pointed end. It is a peculiar feature (Fig. 3.4) that a droplet 0.1 μ long may be extended into "threads" several micra long in the course of the change. It is impossible to explain this drawing out by cell elongation, since this is quite inadequate to give the order of extension required. A view, compatible with the microscopic evidence, is that the modified form is pushed out from the droplets by growth (crystallisation) at the surface of the droplet.

As mentioned earlier it is difficult to be sure from a study of cross-sections, whether the modified form is filamentous or sheet-like. A protein of the α-type in the form of thin sheets has been extracted by Rudall from an insect source (4), although its formation bears little resemblance to that described here.

As the transformed trichohyaline condenses, it shows in longitudinal sections a remarkably parallel system of thin filaments (or sectioned sheets) spaced less than 100 A apart. The appearance of this form coincides with the sudden appearance of anisotropy in the Henle layer and later in the Huxley and sheath cuticle layers.

EXPLANATION OF PLATES

PLATE 57

Fig. 3.1. An electron micrograph of a longitudinal section of the peripheral regions
of the mid-bulb showing the early development of the inner root sheath. The cells
have already developed extensive adhesive contacts (C) and these areas of contact are
spreading. Both the presumptive Henle cells on the right and the Huxley cells in the
centre have commenced the formation of trichohyaline, which is seen as rounded
structureless bodies (T) in several of the cells.

Clusters of dense particles (P), assumed to contain ribonucleic acid, are common; a
number of other less well defined agranular vesicles and various mitochondria (M)
may be seen. The trichohyaline near the top right corner is already partly transformed.
× 24,000.

Fig. 3.2. A longitudinal section passing almost tangentially through the Huxley
layer immediately above the bulb constriction, showing the accumulations of tricho-
hyaline (T) at this level. The transformation into the fibrous form has begun and fine
filaments (F) may be seen in many cells.

At the top left corner may be seen the edge of the Henle layer (He), which has al-
ready been completely transformed. The fibre axis runs obliquely across the section
from the lower left to the upper right corner and many of the trichohyaline droplets
are elongated in this direction. N is a nucleus. × 16,000.

formation occurs at the level of the neck of the follicle. Synthesis and transformation in the cells of Huxley and the sheath cuticle lag behind the similar events in the cells of Henle. The transformation does not begin until the lower prekeratinous zone in the Huxley and cuticle cells.
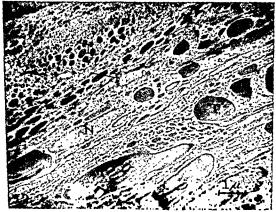
5. The amorphous-fibrous transformation occurs rapidly cell by cell and involves the conversion of all the trichohyaline droplets. In longitudinal sections the birefringent modification can be seen extending from the droplets in both directions parallel to the axis of the hair. In cross-sections the images of the transformed material are difficult to interpret. They may be seen as sections of corrugated sheets ($\sim$100 A thick) or condensed fibrils $\sim$100 A in width.

6. At the same time that the trichohyaline transforms, the spacing between the cell membranes increases and a dark deposit appears centrally between them. This membrane complex, and the similar complex of the hair cuticle cells described in Part 2, may be specialised formations whose purpose is to hold the hardened cells together.

REFERENCES

1. Abercrombie, M., and Heaÿsman, J. E. M., *Exp. Cell Research*, 1953, **5**, 111.
2. Auber, L., *Tr. Roy. Soc. Edinburgh*, 1952, **62**, 191.
3. Montagna, W., The Structure and Function of Skin, New York, Academic Press Inc., 1956.
4. Rudall, K. M., Protein Ribbons and Sheets, *in* Lectures on the Scientific Basis of Medicine, (F. R. Fraser, editor), London, Athlone Press, 1957, **5**, in press.
5. Waddington, C. H., The Principles of Embryology, London, George Allen & Unwin, Ltd, 1956.
6. Weiss, P., *J. Embryol. and Exp. Morphol.*, 1953, **1**, 182

Fig. 3.3. Three comparison micrographs of the same field show the transformation of amorphous trichohyaline of the Henle layer into the fibrous form. The light micrograph (a) is a section stained with Heidenhain's iron haematoxylin and the micrograph (b) is a part of the same field between crossed polaroids to show the birefringent regions In the cortex (1) on the left may be seen the birefringent fibres of keratin and numerous pigment granules. The imbricate cells of the cuticle of the hair (2) are not birefringent The transformation of Henle's layer (5) may be seen to occur suddenly at T. The droplets of the amorphous form are disappearing in one cell and the next cell is highly birefringent. An electron micrograph (c) of precisely the same area makes it possible to see the details of the change more clearly. A lesser degree of birefringence in the sheath cuticle (3) and in Huxley's layer (4) indicates that some transformation has occurred in these regions also (a) and (b) $\times$ 2,500; (c) $\times$ 30,000

PLATE 59
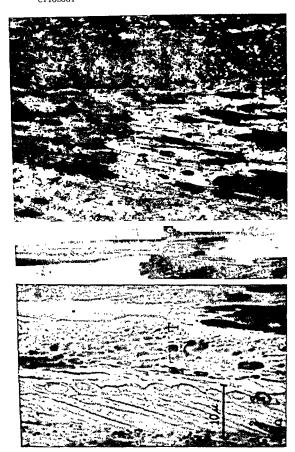
FIG. 3.4. Longitudinal section of part of a Henle cell showing details of the transformation of the amorphous trichohyaline (T) into the fibrous form (F). Stained with 1 per cent phosphotungstic acid in 70 per cent alcohol for 1 hour immediately after fixation.

The amorphous trichohyaline droplets have become lenticular in shape with their long axes parallel to the axis of the follicle and the transformed filaments may be seen extending from the pointed ends. × 80,000.

FIG 3.5 (a) Part of a longitudinal section of a Henle cell immediately after the greater part of the trichohyaline has transformed and the cell is condensing. Stained with phosphotungstic acid as described for Fig 3.4. × 80,000.

(b) Cross-section at the same level as (a) showing the appearance of the transformed trichohyaline in cross-section. × 160,000.

(Birbeck and Mercer Human hair follicle Part 3)

PLATE 60
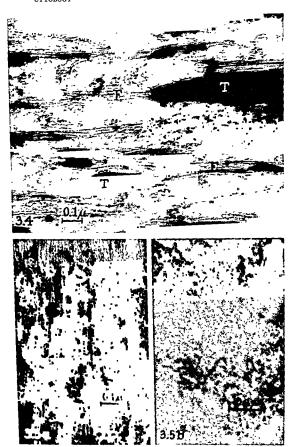
FIG. 3 6. Cross-section of part of the inner root sheath showing fully condensed cells
On the left is the gap which forms between the hair cuticle (not shown) and the cuticle
of the inner root sheath (C) Two overlapping sheath cuticle cells may be seen in the
sheath cuticle with well defined surface membranes. The intercellular cement is rela-
tively dense, but the denser central line, well developed elsewhere, is here only faintly
visible The dark vertical line parallel to the boundary of the cuticle and the Huxley
cell (Hu) is a fold in the section. The cell membrane complex between the Huxley
cell (Hu) and the Henle cell (He) is of the "three line" type shown enlarged in Fig 3 8
Although the total width of the complex is rather constant, certain irregularities, such
as dilations (D) and broader central lines at (B), suggest that the structural pattern
is not rigidly defined. The break in the membranes at G suggests a direct continuity
between the cytoplasm of the two cells.

The trichohyaline in each of the cells has been fully transformed into the fibrous
form and the fine structure in cross-section is similar to that shown enlarged in Fig 3 5
(b) The density of the condensed trichohyaline decreases on proceeding from the
Henle cells to the cuticle and the "texture" grows finer in detail. × 24,000.

FIG 3 7. A cross-section showing the contact between two Henle cells at the level
of the trichohyaline transformation showing details of the development of the inter-
cellular deposits. Transformed trichohyaline is seen at T. The two plasma membranes
(M) have thickened and dilated Localised deposits of ill defined dense material may
be seen on the cytoplasmic side of the membranes. Between the membranes, the central
dense layer (D) is partly formed. × 120,000.

FIG 3 8 The surface membrane complex of the cells of the Huxley layer in which
may be seen the two plasma membranes (M) and the dense deposit or layer (D) be-
tween the plasma membranes A less dense material occupies the remainder of the
intercellular space This formation develops directly from that shown in Fig. 3 7. In
the cytoplasmic space may be seen sections of transformed trichohyaline and of two
mitochondria. The cells at this level are solid. × 120,000

(Birbeck and Mercer Human hair follicle Part 3)
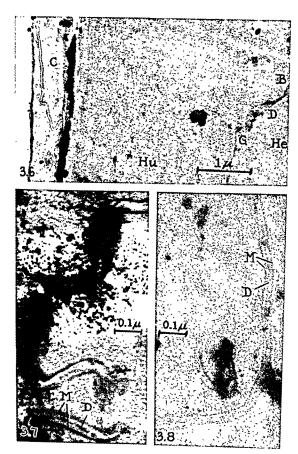
The Use of the Electron Microscope to Control Preparation of Cellular Constituents

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FOR a number of years biochemists have investigated cell fractions separated by centrifugation. The morphological identity of these fractions has sometimes been determined by light microscopy, although the fractions are usually characterised by their biochemical activity. By observing these fractions with the electron microscope, using the sectioning method, and by comparing them with the cell components in whole tissue, it is possible to have a better control of the separation. It is also possible to improve the method of separation, and some of the general methods together with particular methods for nuclei and mitochondria which we have evolved in our laboratory, will be described.

General methods.—The conventional method of separating the cell fractions uses an angle-head centrifuge; with this method fractions must be washed several times by resuspension and resedi-

(Birbeck and Mercer. Human hair follicle. Part 3)

(595)

swelling and the retention of contents increases their sedimentation rate. The medium has no biochemical disadvantages; it does not significantly inhibit any enzymes.

Using this medium particles (fig. 2, inset) have been seen in light mitochondrial fractions which are similar to those described by Kuff *et al.* (6). They are smaller and denser than mitochondria; they have only a single external membrane and a rather granular interior. Duve (4), from the biochemical evidence of enzyme distributions in light mitochondria fractions, has postulated a new cytoplasmic particle, the lysosome in which certain hydrolytic enzymes would be contained.

Discussion.—These results demonstrate how electron microscopy can help the biochemist by giving him an improved method of controlling the purity of the cell fraction. Moreover they also demonstrate how the methods may be improved so that the fractions may be obtained not only in a greater state of purity, but also less damaged by the separation.

The method is of equal importance to the electron microscopist, for it is probably the only way in which he will be able to determine the chemical nature of the fine structures he sees. It is unlikely that histochemistry will ever be possible in electron microscopy, to the same extent as it is in light microscopy. The electron microscopist must there-fore separate the structures he observes without damaging them, and then chemically analyse them. The techniques of separation by centrifugation are examples of such a general method.

REFERENCES

1. BIRBECK, M. S. C. and REID, E., *Biochim. et Biophys. Acta* 20, 419 (1956).
2. — *J. Biophys. Biochem. Cytol.* (1957, in press).
3. DAVISON, P. F. and MERCER, E. H., *Exptl. Cell Research* 11, 237 (1956).
4. DUVE, C. DE, PRESSMAN, B. C., GIANETTO, R., WATTIAUX, R., and APPLEMAN, F., *Biochem. J.* 60, 604 (1955).
5. HOGEBOOM, G. W., SCHNEIDER, W. C., and PALADE, G. E., *J. Biol. Chem.* 172, 619 (1948).
6. KUFF, E. L., HOGEBOOM, G. H., and DALTON, A. J., *J. Biophys. Biochem. Cytol.* 2, 23 (1956).
7. PALADE, G. E., *J. Exptl. Med.* 95, 285 (1952).
8. PHILPOT, J. St. L. and STANIER, J. E., *Biochem. J.* 63, 214 (1956).
9. WITTER, R. F., WATSON, M. L., and COTTONE, M. A., *J. Biophys. Biochem. Cytol.* 1, 127 (1955).

mentation in fresh medium. We have found that an improved separation may be obtained more quickly by using layering or gradient methods in a swing-out head centrifuge. When the medium contains high molecular weight components, which are required to improve the morphological appearance of the fractions, density gradients may be obtained without increasing the osmotic strength of the medium.

Fixation of cell fractions by the standard buffered osmium tetroxide solution (7) is satisfactory; no improvement has been found either by the addition of sucrose or high molecular weight components to the fixative, or by using a solution of osmium tetroxide in the suspending medium as a fixative. A small volume of a resuspended fraction is added to a larger volume of fixative; the fixed suspension is then spun to produce a pellet. The pellet, which should be less than 0.5 mm thick, is treated as a lump of tissue. It is dehydrated and embedded in methacrylate as in the conventional method. The *pellet is oriented on the microtome so that a single section contains all the strata produced by sedimentation; in this way a proper estimate of the distribution of material in the pellet may be obtained.*

A promising variant of these methods has been devised by us in which no attempt is made to isolate separate fractions. The tissue is homogenised in a medium, and the suspension is "layered" over a medium of higher density in a centrifuge tube. Using a swing-out head, the suspension is sedimented to form a pellet about 0.1 mm thick. This will contain "strata" of all components of the suspension separated according to their sedimentation rate. The whole pellet may then be fixed, sectioned and the composition of the various layers determined electron microscopically. As an example of its use, consider the problem of the uptake of a radio-active amino acid in a tissue. The compound is injected, after a certain time tissue is taken from the animal, homogenised and centrifuged by this method. The block is sectioned; a thin section is examined in the microscope and a radio autograph is made from a contiguous thick section. By comparing these, the particular cell fraction in which the radioactive isotope has been taken up may be found. *The method may also be used for the intracellular localisation of other constituents which are still detectable by histochemical methods after fixation.*

Methods for nuclei.—Nuclei, separated by many of various standard methods, have been examined (3). None of these methods gives nuclei of comparable appearance to those in sections of fixed tissue; many methods give nuclei which appear clean in the light microscope, but are badly contaminated by cytoplasmic components when examined in the electron microscope. Nuclei prepared by the method of Philpot and Stanier (8) were found to be particularly interesting. This method, which only gives small yields, consists of centrifugation in a sucrose glycerol medium with the addition of glycero-

Fig. 1. Electron micrograph of a single nucleus in a fraction prepared by the method of Philpot and Stanier. The nucleolus may be seen, but the nuclear membrane is absent.
Inset. A high power micrograph of the nucleoplasm, showing filaments about 100 Å in diameter.

Fig. 2. Electron micrograph of a few mitochondria prepared in the dextran medium. The internal and external membranes may be seen.
Inset. A micrograph at the same magnification of a particle in a light mitochondrial fraction, which might be a lysosome.

phosphate These nuclei (fig. 1) from rat liver are relatively clean of contaminating cytoplasm; this may be partially the result of the loss of the nuclear membrane, to which the cytoplasmic debris tends to adhere. There are, however, numerous small filaments (ca. 100 Å diam.) dispersed throughout the nuclei (fig. 1, inset). These filaments which significantly have the same diameter as nucleo-protein filaments, may be an artifact due to the method of separation; however, it is also possible that they also exist in sections of whole tissue, but are rendered invisible by an interstitial substance, which is lost during separation, possibly as a consequence of the removal of the nuclear membranes.

Methods for mitochondria.—The conventional method for the separation of mitochondria uses 0.25 M sucrose as a suspending medium (5) Mitochondria prepared by this method and by improved methods have been examined by several workers (1, 2, 6, 9). Mitochondria prepared in 0.25 M sucrose are swollen, empty and usually contaminated with microsomal debris. We have described an improved complex medium containing 0.25 M raffinose, 6 % dextran and other additives. Mitochondria prepared in this medium (fig. 2) are not swollen and retain their internal double membranes and other contents. When the centrifugation conditions are carefully controlled it is possible to obtain cleaner mitochondria using this medium. This may be judged both by the lack of microsomal material in the electron micrographs and also biochemically by their low R.N.A. assay. Although the higher density and viscosity of this medium would appear to make high centrifugation speeds necessary, this is not so for mitochondria as the absence of

Fig. 2. Electron micrograph of the mid-bulb region of the follicle showing early cell contacts and (probably) contact spread.

The cells of the matrix contain many mitochondria, many agranular vesicles and large numbers of the dense particles thought to contain ribonucleic acid (3). There are none of the specialised intracellular inclusions which appear later at higher levels in the follicle. The surfaces of the cells are very convoluted and intercellular gaps are common Numerous small finger shaped pseudopods project from the surfaces and often penetrate deeply into neighbouring cells. Close contact between the cells is limited to a few small areas where the two plasma membranes are parallel and separated by a distance of about 120–150 Å. We conclude therefore that the surface membranes of the undifferentiated cells are flexible and in active motion. Their adhesion is small and contact is both temporary and limited in area. The space between the membranes at these "adhesive contacts" is not by any means empty. A material of rather poor electron scattering power is observed both between the closely opposed surfaces and also spread out over the immediately adjacent surfaces. We suppose that this material is the cement responsible for the adhesion of the surfaces. Very little can be deduced concerning its chemical nature. The very slight reaction with the osmium fixative suggests that it is not protein or-lipid in nature, but that it

might be polysaccharide. In view of the important role we shall assign to it in morphogenesis, it is most regrettable that more is not known.

When the cells further along the follicle are examined the importance of cell contacts becomes apparent (figure 2). The areas of contact spread and, *in a zipper-like fashion, draw the cells together* with the result that intercellular gaps are closed and the surface convolutions are smoothed out (figure 1). This development does not take place uniformly throughout the cell population. Contact spread occurs first in the cylinder of cells, which will become the cuticle of the hair, and follows rapidly in those cells, between the cuticle and the outer sheath, which form the three layers of the inner sheath. However, *in the cortex, the central cylinder of cells*, contact spread does not occur until much later; the cells remain united only locally and intercellular gaps are common.

Contact spread appears to have important morphogenetic consequences, *some of which can be deduced from the appearance of the cells of the various layers in the mid and upper bulb. The cells* of the cuticle assume an important place in all subsequent developments because between them adhesive contacts appear early, spread more extensively and seem to be stronger. The cuticle stands out from the surrounding cells partly because of the density of its membranes and also because of the cuboidal shape of the cells in longitudinal section. It appears that the surface adhesion between these cells is great *enough to actually modify their shape.* By drawing the membranes of contiguous cells together in zipper fashion (4) the mass of previously rounded cells is converted progressively into a columnar layer (figure 1), which divides the advancing stream of cells into two domains whose subsequent developments are in striking contrast. Inside the cuticular "barrier" is the cortex where the characteristic product of cell synthesis is fibrous keratin; outside we find the inner root sheath where amorphous trichohyaline is the typical product.

In the cortex contacts are localised and surface activity persists at least as high as the follicular constriction. *The melanocytes,* which form the pigment of the hair, are situated largely near the tip of the papilla, *and appear able to take advantage of the* weak adhesion between the cortical cells to extend their long pigmented processes throughout the cortical space. The absence of gaps between the cuticle cells prevents the processes *penetrating the cuticle* and the inner root sheath beyond. The actual incorporation *of the pigment granules into the* cortical cells appears to be a consequence of the continued surface activity of these cells *for we can* see small pseudopods enveloping the ends of the pigmented processes in a manner suggestive of phagocytosis. Occasionally bundles of granules still enclosed in membranes have been found in cortical cells (1).

The Role of Cell Membranes in Morphogenesis

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THE study of morphogenesis by electron microscopy is likely to prove a formidable undertaking However, the labour can be lessened by the choice of an appropriate system in which there are present not only various examples of differentiated cells but, at the same time, cells in different stages of development. It is a further advantage to have the different stages distributed in the tissue in an easily recognised manner

Systems of cells satisfying these demands are not common. One, which is admirable for the purpose, is the follicle of the growing hair of the mammalian skin. Here we find at the end of a small tube, the outer root sheath, dipping down into the skin, a germinal matrix producing a steady stream of cells which, passing along the tube, differentiates into the six concentric cylinders of cells which form the hair and its enveloping sheaths In the distance of a few 100 μ of the tube we find cells in the following stages· dividing cells, undifferentiated cells, cells in early differentiation and differentiated cells engaged in synthesis of their characteristic products It is thus possible in a single electron microscopic section to find examples of cells in all stages of their development arranged in a linear sequence.

Taking advantage of this situation, we have examined in hair follicles—fixed, embedded and sectioned by the now standard procedures—the events associated with early differentiation, which occur in the mid and upper regions of the bulb (see figure 1). By comparing the cells of the undifferentiated matrix with those, a few cell diameters further along the follicle which show definite signs of differentiation, we have been led to suspect that the contacts between the surfaces of the cells play a leading role in morphogenesis. (See also reviews (5) and (6))
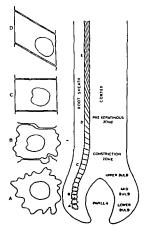
Fig 1 Diagrammatic representation of the behaviour of cell membranes and morphogenetic developments in the hair follicle with reference to the hair cuticle. In the outline of the follicle, shown in the centre, the changes in the cuticular formation and the several zones of the follicle are indicated On the L.H S is shown a series of cells (A to D) in which cell contacts are shown developing, spreading and deforming the cells

(599)

Fig. 2. Electron micrograph of the mid-bulb region of the follicle showing early cell contacts and (probably) contact spread.

might be polysaccharide. In view of the important role we shall assign to it in morphogenesis, it is most regrettable that more is not known.

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The cells of the matrix contain many mitochondria, many agranular vesicles and large numbers of the dense particles thought to contain ribonucleic acid (3). There are none of the specialised intracellular inclusions which appear later at higher levels in the follicle. The surfaces of the cells are very convoluted and intercellular gaps are common. Numerous small finger shaped pseudopods project from the surfaces and often penetrate deeply into neighbouring cells. Close contact between the cells is limited to a few small areas where the two plasma membranes are parallel and separated by a distance of about 120–150 Å. We conclude therefore that the surface membranes of the undifferentiated cells are flexible and in active motion. Their adhesion is small and contact is both temporary and limited in area. The space between the membranes at these "adhesive contacts" is not by any means empty. A material of rather poor electron scattering power is observed both between the closely opposed surfaces and also spread out over the immediately adjacent surfaces. We suppose that this material is the cement responsible for the adhesion of the surfaces. Very little can be deduced concerning its chemical nature. The very slight reaction with the osmium fixative suggests that it is not protein or·lipid in nature, but that it

Fig. 3. Complex intercellular membranes between the cuticle cells.

Fig 4. Complex intercellular membranes between the Henle cells (inner root sheath)

The dense, closely adhering membranes of the cuticular cylinder not only appear to form a barrier separating the domain of keratin formation (cortex) from the domain of trichohyaline formation (inner sheath), but appear also to prevent the cuticle itself from acquiring sufficient raw materials. For although syntesis is rapid in the cortex and the inner root sheath, in the cuticle itself, synthesis is delayed until above the constriction of the follicle when a form of amorphous keratin with a high cystine content appears.

A discussion of the structure of keratin and trichohyaline will be found elsewhere in this volume (2). These two proteins are responsible for the hardened and fibrous texture of the cells of the hair and sheath which develops rapidly above the follicular constriction. Simultaneously with the development of these intracellular products the intercellular structures also undergo a remarkable development. Until this level the adhesive contacts have consisted simply of the two plasma membranes and the intervening cement layer of 120–150 Å thick. As the cells of the cuticle and sheath fill with protein, the plasma membranes suddenly dilate to a distance of 300–400 Å and a series of complex layers form between them (figs. 3 and 4). The nature and function of these intercellular layers are obscure; possibly they serve to hold together the hardened cells. The dense layers (one in the sheath cells and two or three in the cuticle) may be deposits of an electron dense tanning agent in a pervading less dense cement of the type described below. In the cortex the membranes also dilate and finally become cemented together but we have not found a layered structure.

REFERENCES

1. BIRBECK, M. S. C. and MERCER, E. H., *Exptl. Cell Research* 10, 505 (1956).
2. — These Proceedings, p. 159.
3. PALADE, G. E., *J. Biophys Biochem. Cytol.* 1, 567 (1955).
4 SCHMITT, F O., *Growth* 5, 1 (1940).
5 WADDINGTON, C H, Principles of Embryology. Allen & Unwin, London, 1956.
6. WEISS, P., *J. Embryol. Exptl. Morphol.* 1, 182 (1943).

Fig 3. Electron micrograph showing fibrils of keratin appearing in a cortical cell in the upper bulb region (longitudinal section). Magnification × 20,000 Fig 4. A cross-section at high magnification of a fibril showing the component keratin filaments (light) on a dark ground—the cystine-rich matrix. Magnification × 150,000.

without any evidence of a non-fibrous precursor. Filaments can be detected electron microscopically in the bulb cells at a level below which the birefringence is strong enough to be demonstrated. Such filaments are oriented parallel to the fibre from their first appearance. These observations clearly show that the keratin is not synthesised as an amorphous precursor which is converted into a fibrous form by its passage through the narrow neck of the follicle.

In the upper bulb, where the rise in birefringence takes place, the cells rapidly fill with filaments (fig. 3) and condense to form the rather definite structures recognisable in the light microscope as fibrils (0.1–0.2 μ in diameter). At this level there is sufficient material present to enable an x-ray diffraction photograph to be made and a typical α-type pattern results (fig. 1). There is therefore little doubt that the long fine filaments are the structures responsible for this x-ray pattern which is of such interest to crystallographers (2).

Cross-sections of the condensed fibrils (fig. 4), show that the filaments are embedded in a material which, after osmium fixation, has a greater electron scattering power than the filaments themselves, i.e. the filament sections appear light on a dark ground. The most probable interpretation is that the osmium is here acting as a specific stain for cystine (cysteine) and that the S sites are concentrated in the interfilamentous regions. Since chemical analysis of dissolved hair (1) shows the presence of a fibrous component (α) with a low S content and an amorphous component (γ) with a higher S content, we suggest that the α-component be identified with the fine filaments and the γ with the interfilamentous cement. Fibrous keratin is thus seen to be a complex of "filaments plus matrix" rather than a single entity.

An unsolved question is the contribution to the observed birefringence of intrinsic and form factors Attempts to determine these contributions by the standard method of immersing the hair in a series of liquids is not possible, since these invariably fail to penetrate. Liquids, which do penetrate the hair, either react with it chemically or swell the entire structure. In either case the double refraction falls, usually irreversibly, to a low value. The structure of fine filaments embedded in a highly cross-linked matrix suggested by electron microscopy may explain these results. The hair is certainly a Wiener body, i.e. a system of oriented rodlets embedded in a matrix, probably with different optical constants, and there is likely to be a form contribution. But, since the matrix is by far the more cross-linked component, suitable imbibition liquids penetrating the matrix alone probably do not exist.

REFERENCES

1. ALEXANDER, P and HUDSON, R. F, Wool, Its Chemistry and Physics. Chapman and Hall, London, 1954
2. ASTBURY, W. T., Proc. Roy. Soc B 134, 303 (1947). Review.
3. MERCER, E. H., Biochim. et Biophys Acta 3, 161 (1949).
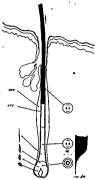
Fig. 1. Diagram of the hair follicle showing the development of the fibrous structure of the cortex. On the right-hand side is shown the rise in birefringence (Δn), in the centre the x-ray diffraction patterns at several levels are indicated in circles, on the left-hand side the changes in cell shape.

The two birefringent systems are well defined when the follicle is viewed between crossed polaroids and measurements (3) show in the cortex a continuous rise in birefringence at the level of the follicular constriction. In the Henle layer of the sheath the change from the isotropic to the birefringent state occurs in a single sudden step, suggesting a rapid transformation of the cell contents. A slower rise occurs in the Huxley layer and the sheath cuticle. The comparison of precisely the same fields in the polarising microscope and the electron microscope

has enabled the birefringence to be correlated with structure.

The inner root sheath.—This correlation is most complete in the Henle layer of the inner root sheath. By examining in the electron microscope the area in which birefringence first appears, we can see in some detail the formation of the fibrous form. The rounded, structureless, dense bodies, which appear lower down in the cytoplasm of the sheath cells and rapidly aggregate to form the large droplets recognisable in the light microscope as trichohyaline, are seen transforming into fibrils. The details of the actual transformation are shown in fig. 2. Fine filaments (about 100 Å diameter) can be seen extending in both axial directions from the lenticular shaped droplets of the amorphous form. The transformation occurs entirely intracellularly and differs from anything previously described, although the formation of fibrils from solutions of corpuscular molecules is well known. It would seem that the filaments "crystallise" out on the surface of the solid droplets and are pushed away from the surface by their continued growth. The nearest physical analogue would be the growth of single crystals from the molten state. Little is known of the chemical composition of trichohyaline to assist our speculations and the amounts present in the hair are too small to make it possible to examine it by x-ray diffraction.

In the other layers of the sheath (Huxley and sheath cuticle) a similar transformation occurs. However, it takes place more slowly and the amorphous and fibrous forms can be seen together, in the same cells, for a distance of several hundred microns above the constriction.

The cortex.—A very different course of events occurs in the cortex. Here the fibrous form appears directly in the form of fine filaments (60–80 Å in width)

Fig. 2. Electron micrograph of a portion of a Henle cell of the inner root sheath at the point of transformation of the elongated trichohyaline droplets into filaments. (Longitudinal section.) Magnification × 73,000.

The Fine Structure of Keratin

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THIS article is a sequel to an earlier one [11], in which the connection between the cellular structure of animal hairs and their chemical heterogeneity was discussed. Hairs consist entirely of dried cells, in many of which the normally occurring constituents of living matter are still present in an altered form along with the extensive deposits of hardened keratin. The "cellular remnants," consisting of nuclei, cell membranes, and less well defined cytoplasmic inclusions, amount to about 10% of the dry weight of the fiber. These may be referred to collectively as the *non-keratins* to distinguish them from the principle constituent, the protein *keratin*.

The word "keratin" should not be regarded as a *name for a single well defined substance*, but rather for a class of intracellular proteins which have in common certain properties of insolubility, stability, and hardness due to a high content of the cross-linking amino acid, cystine. The keratin of the cuticle, for instance, differs from that of the cortex in not being fibrous; in the cortex itself, the fibrous keratin may differ in solubility and stability from cell to cell. When, as in wool and other curly and crimpy fibers, there is a relation between the fiber shape and the geometrical distribution of stable and less stable keratin, this type of heterogeneity assumes a particular importance in determining the behavior of the fibers [7, 8, 11].

In the present paper we wish to discuss the fine structure of the keratin itself, i.e., the structural details of a size smaller than can be resolved by the light microscope but larger than those responsible for the wide angle X-ray diffraction pattern (< 10Å). Structures of this order (10–1000Å) are most easily studied electron-microscopically; our results were obtained by an application of the method of thin (or ultra) sectioning. It will be shown that at this level fibrous keratin may be considered to possess a duplex structure — a system of fine filaments (diameter ~ 60Å) held together by an amorphous cement. The fibrous elements may be more or less completely fused according to the amount of cement present.

Some of this work, particularly relating to the formation of keratin, has been described previously [3, 5, 14]. The present paper will be mainly concerned with its relevance to the structure of keratin itself, with special reference to wool.

Materials and Methods

The method of preparing thin sections (0.05–0.02μ) of biological materials for electron microscopy need not be described here, as it is already a standard procedure subject only to minor variations in different laboratories [16, 17]. We have imbedded and sectioned both fully hardened hair and wool (or derivatives of these) and partly formed hair in follicles of the skin. Fully hardened hair has not proved easy to section, and, unless chemically modified, the sections, when obtained, were not very informative. However, the fully formed but incompletely keratinized hair in the follicle is a more amenable material; it provided most of the results to be described below. Except in the case of the hardened hair, it was necessary to fix the material before imbedding. The hardened hair is, in a sense, already fixed, and fixation has some resemblances to keratinization. The only suitable fixative for the hair root is the buffered osmium tetroxide solution (1% OsO₄ at pH 7.2) described by Palade [16]. Buffered formaldehyde preserves many of the large scale structures, but is inferior to the osmium fixative for fine detail. The same conclusion has been reached by electron microscopists who have studied the fixation of other tissues [16].

Hair roots or wool roots were plucked, immersed immediately in the osmium fixative for periods of 2–4 hr., washed, dehydrated, and imbedded in methacrylate or epoxide polymers. With certain partly disintegrated derivatives of hair, a fixation in buffered formaldehyde solution (10% formaldehyde at pH 7) was satisfactory. Fiber fragments, e.g., from enzymatic disintegration or chemically extracted fibers, were fixed in formaldehyde solution for 24 hr., washed, dehydrated, and imbedded.

Electron Micrographs of Cross-Sections of Protein Monolayers

ELECTRON micrographs of cross-sections of protein monolayers have been obtained in the following way. A monolayer of protein, a commercial sample of egg albumen, was spread at the surface of water contained in a small Langmuir type trough. This layer was then compressed and crumpled by advancing a barrier coated with paraffin wax towards a second retaining barrier. When the two barriers were about a centimetre apart, the crumpled film became visible at the interface and was lifted from the water as a frail thread by means of a wire yoke. Portions of this thread were prepared for cross-sectioning by dehydrating in an alcohol series and embedding in an epoxide resin[1]. It was found advantageous to 'stain' the specimen first by treating it for a short time in a buffered osmium tetroxide solution. The thread, darkened and hardened by this treatment, was easier to handle and to see by eye in the block and also in the electron microscope. Very thin sections were cut from the block at right angles to the length of the thread by the standard procedure for electron microscopy[2] and examined in a Siemens 'Elmiskop I' microscope.

The cross-section of the crumpled sheet of protein appeared as a thin, dense, highly convoluted line (Fig. 1). Since the thickness of the best sections (about 200 A.) was still some ten times that of the monolayers (10–20 A.), the films viewed end on would everywhere reveal their true thickness. The thinnest portions observed in osmium-fixed material were in fact about 50 A. thick, and some of these appeared double, suggesting either that the osmium was deposited on each side of the protein film or that more than a simple monolayer was present. Occasionally a contaminating layer was seen adsorbed on one side of the film and a pinched-off tubule usually enclosed some excess protein.

This work is part of an attempt to prepare artificial models of cell membranes. Multiple layers and tanned layers are also being examined. It is remarkable how closely some of the chance structures in these crumpled films resemble sections seen in electron micrographs of such organelles as the mitochondrion and the Golgi complex.

Fig. 1. Electron micrograph of a cross-section of a crumpled monolayer of egg albumen stained with osmium tetroxide

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     May 13.

[1] Glauert, A. M., Rogers, G. E., and Glauert, R. H., *Nature*, **178**, 803 (1956).
[2] See accounts given in Proceedings of the International Conference on Electron Microscopy. London 1954 (1956)

soluble than the other membranes. It is evident that one of the changes occurring during the hardening of epidermal structures is a thickening and chemical modification of the cell membranes.

Figure 2 shows the relations of the several layers of the cuticle and certain cuticular derivatives as they appear in section, and is essentially the same as that deduced earlier [10]. It will be noticed that there is no layer between the cortical cells and the cuticle cells (i.e., no "subcutis" or "between membrane"). All the various sheetlike or tubular structures observed in chemically broken down hair can be accounted for in terms of the several layers of the cuticle itself or of the membranes of the cortical cells.

Fine Structure of Cortical Keratin

Within the cortical cells themselves are found the solid condensed mass of fibrous keratin whose detailed structure has previously been studied electron-microscopically by methods which involved the dis-

integration of the fiber into thin fragments. Such methods showed that fibrous units of two orders of size could be distinguished (see Review in [10] and [14]); large fibrils visible in the light microscope ($\sim 0.2\mu$ in diameter) and, within these, finer filaments approximately 100A wide. A study of thin cross sections confirms these findings in essentials, but gives a much more complete and satisfactory view.

It has proved informative to compare cross sections at successive levels in the growing follicle which differ in the degree of consolidation of the keratin, in order to obtain an insight into the nature of one keratinization [5]. An example of such a section is given in Figure 3. A higher resolution micrograph of a portion of a field, such as that of Figure 3, is shown in Figure 4.

First of all, one notes the existence of the large fibrils (seen here in cross section) of a considerable range of size and of cross-sectional shape. The high
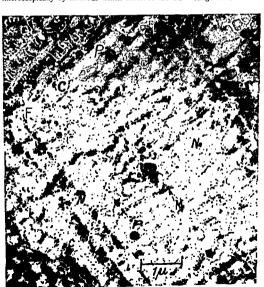
Fig. 3. An electron micrograph of a cross section of a hair in the upper keratinous zone of a human hair follicle, showing portion of the cortex. Two nuclei are seen at N and fibrils in cross section as at F. A cell membrane may be found at M. P is a pigment granule C is the edge of the cuticle.

The electron microscopes used were a Siemens Elmiskop 1 and a Philips 100.

Results and Discussion

The Histological Level

Before proceeding to describe the new results of high resolution microscopy, it should be mentioned that the use of the electron microscope at low magnifications ($<10,000\times$) has confirmed the findings of the earlier work [11] mentioned above, which was based on light microscopy. The cellular character of the hair or wool, which is usually difficult to appreciate in the light microscope, is immediately obvious in cross sections in the electron microscope. The cortex in cross section is seen as a condensed mass of cells whose faces meet in complicated re-entrant or interlocking surfaces [14]. Since the cells are solid, these interlocking surfaces must contribute to the strength and coherence of the structure. The two cell membranes at each interface and the nuclear residues may also be seen.

The conclusion reached earlier [12] that the residues, after extraction of wool with alkalies (or after the extraction of oxidized wool with ammonia), consisted of cell membranes, nuclei, and other ill defined nonkeratins was confirmed by examining sections such as that shown in Figure 1, which shows this residue. The residue was previously described as a tubular structure placed between the cuticle and cortex [1].

Some new facts have emerged concerning the cuticle. That the cuticle possesses in effect a laminar structure — a very resistant external membrane (epicuticle), a keratinous layer (exocuticle), and an inner, nonkeratinous layer (endocuticle) — was deduced from an examination of fragments of disintegrated fiber some years ago [10, 13]. Sections of such fragments and also of extracted wool confirms these deductions and show that the inner layer (endocuticle) really consists of the modified remnants of the cellular apparatus proper to these cells [5]. The cell membranes of cuticle and cortex are insoluble in keratinolytic solvents for unknown reasons; the external cell membrane of the cuticle cells (epicuticle) appears thicker, more dense, and more in-

Fig. 1. An example of the use of the method of thin sectioning for electron microscopy to elucidate the results of the chemical extraction of a wool fiber. The fiber has been oxidized by peracetic acid and extracted with ammonia [1]. The keratin has been entirely removed; the residue consists of cell membranes M, nuclei N, and cytoplasmic debris D. The external membrane E of the cuticle cells, which forms the epicuticle, is somewhat more dense than the internal membranes. The cytoplasmic debris of the cuticle cells forms a well defined layer, the endocuticle ED (see Figure 2).

Fig. 2. The structure of the cuticle cell and of certain chemically extracted derivatives of that cell as deduced from thin sections. (A) portion of an intact cell showing (1) the external cell membrane (epicuticle), (2) the keratinized layer (exocuticle), (3) the inner layer (endocuticle) consisting of the condensed cellular apparatus which is not keratinized and is insoluble in keratinolytic solvents, (4) the nucleus, and (5) the inner cell membrane, (B) the membrane of the Allworden sacs, (C) the residue of the cuticle cell remaining after tryptic digestion, and (D) the cuticular residue remaining after the fiber, oxidized by peracetic acid, is extracted to remove the keratin [2].

the fixative, leading to strongly electron scattering deposits of the lower oxides of osmium in the cystine-rich regions. In brief, the osmium is an "electron-stain" for cystine. The fact that the interfilamentous material is more dense than the filaments must mean, therefore, that there is more cystine in the cement than in the filament themselves.

We are thus led to the filament plus cement model for the fine structure of keratin, which has already been suggested on other grounds [1, 10, 13]. In Figure 5d this model is shown diagrammatically. The filaments are the fibrous units of the complex and must be the structures responsible for the wide angle X-ray diffraction pattern (the α-pattern) studied by Astbury and his collaborators [2]. These units may be referred to as α-filaments for this reason.

The cement is probably a protein of different composition from the filaments, particularly in respect of the frequency of cystine residues, which may be as high as 1 in 2 or 3. Its extensively cross-linked character possibly explains why it is not fibrous. Keratinization would thus seem to be a process whereby an insoluble complex is formed by imbedding a system of rather soluble filaments in a very insoluble cement. Degrees of keratinization can be conceived to arise from differences in the amount of cement present. Where the keratinization is more extensive, e.g., in the paracortex of wool fibers, the amount of the cement protein is probably increased, leading to a somewhat higher sulfur content.

Chemical Extraction of Keratinized Fibers

This model is of value in explaining what might be expected when attempts are made to dissolve keratin. Assuming all the cystine cross-linkages are broken, one may expect to obtain derivatives of both filaments and cement. This seems to be the case when peracetic acid used to oxidize the cystine, as has already been suggested by Alexander [1]. Other solvents may extract more or less of the two components in various states of breakdown, but in any case an electron microscopic study of thin sections of the extracted material (Figure 1 is an example) would be necessary to reveal the precise nature of the attack. This kind of collaboration between electron microscopist and chemist seems essential in further work on the composition of all heterogeneous fibers.

The Organization of the Fine Filaments Within the Fibrils

In the center of the fibrils shown in Figure 5 it will be seen that the filaments are packed in an approximately hexagonal array; in other areas, particularly towards the edges of the fibrils, they appear to be arranged in a series of parallel rows, and the individual filaments are hard to make out. In micrographs, such as Figure 6, spirals and whorls may be seen clearly. These structures, which are not clearly developed in earlier aggregates of filaments formed lower down in the follicle, appear to be a form of consolidation of the structure brought about by the adhesive effect of the interfilamentous cement. The reason why rows or lines of filaments are seen rather than single filaments is not immediately obvious, nor do longitudinal sections clear the matter up. A likely explanation is that, where whorls are seen in cross section, the fibrils are also slightly twisted around their long axis. When cross-sectioned (see Figure 5c), only those filaments which are nearly at right angles to the section will be seen end on; others will appear as dots; others will be tilted to the plane of the section; their images will partly fuse and appear as parallel lines. Twisted fibrils have in fact

Fig. 5. A diagram to illustrate the structural features of cortical keratin as described in the text. (A) two cortical cells from wool in cross section showing nuclei and fibrils. On the left is an ortho-type cell in which the fibrils remain separate. On the right is a para-type cell in which an extensive lateral fusion of the fibrils has occurred. This situation prevails in human hair, (B) two modes of arrangements of the spiral structures within the fibrils seen in cross sections of hair (See Figure 6), (C) suggested twisted arrangement of the filaments in a fibril which can lead to appearances such as B in cross section and which can unroll and lead to sheets of filaments, and (D) the "filament plus cement" model for fibrous keratin. The α-filaments (large dots, diameter about 60Å) are imbedded in a sulfur-rich cement (fine dots—γ).

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Summary

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4. The outer cell membrane of each cuticle cell is
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tutes the special membrane referred to as the epi-
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nated. an outer (nonfibrous) layer of keratin and an
inner layer, a residue of the cell apparatus, which,
although very insoluble, is not keratin.

5. The fibrous keratin of the cortical cells consists
of condensed bundles or fibrils of fine filaments (α-
filaments, width ca. 60Å). Further, the large bun-
dles (or fibrils) may be extensively fused laterally
to give massive irregular aggregates ($0.05-0.2\mu$).

6. This condensation is more extensive on the
para side of wool fibrils than on the ortho (outer face
of crimp wave), and explains the greater insolubility
of the paracortex.

7. The α-filaments are held together in the fibrils
by a cement which does not appear to be fibrous and
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same cement holds the fibrils together when they
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8 The protein of the cuticle is not fibrous and ap-
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Acknowledgments

Fig. 6. Cross section of the cortex prior to the final con-
densation, showing the spiral arrangement of the filaments
within the fibrils A similar appearance is produced in the
final hair if it is reduced and stained with the osmium fixa-
tive. C is the edge of the cuticle; P is a pigment granule.

Literature Cited

1. Alexander, P., and Hudson,, R. F., *Wool: its Chemistry and Physics*, Chapman and Hall, London (1954).

2. Astbury, W. T., and Woods, H. J., *Phil. Trans. Roy. Soc.* 232A, 333 (1933).

3. Birbeck, M. S. C., Mercer, E. H., and Barnicot, N. A., *Exper. Cell Res.* 10, 505 (1955).

4. Birbeck, M. S. C., and Mercer, E. H., *Nature* 178, 985 (1956).

5. Birbeck, M. S. C., and Mercer, E H., *J. Biophysic. and Biochem. Cytol.* 3, No. 2 (1957).

6. Glauert, A. M., Rogers, G. E., and Glauert, R. H., *Nature* 178, 803 (1956).

7. Fraser, R. D. B., and Rogers, G. E., *Biochim. Biophys. Acta* 12, 484 (1953).

8. Horio, M., and Kondo, T., TEXTILE RESEARCH JOURNAL 23, 373 (1953).

9. Jeffreys, G. M., Sikorski, J., and Woods, H. J., *Proc. Internat. Wool Textile Conference, Australia*, F, 130 (1956).

10. Lindberg, J., Mercer, E. H., Philip, B, and Gralén, N., TEXTILE RESEARCH JOURNAL 19, 673 (1949).

11. Mercer, E. H., TEXTILE RESEARCH JOURNAL 23, 388 (1953).

12. Mercer, E. H., *Nature* 172, 164 (1953).

13. Mercer, E H., and Rees, A. L. G., *Aust. J. Exp. Biol. Med. Sc.* 24, 147, 175 (1946).

14. Mercer, E. H., Farrant, J. L., and Rees, A. L. G., *Proc. Internat. Wool Textile Conference, Australia*, F, 120 (1956).

15. Mercer, E. H., TEXTILE RESEARCH JOURNAL 22, 477 (1952).

16. Palade, G. E., *J. Exp. Med.* 95, 285 (1952).

17. Sjöstand, F. S., "Electron Microscopy of Cells and Tissues," in *Physical Techniques in Biological Research*, Vol. III, G. Oster and A. W. Pollester, Ed., Academic Press, New York (1956).

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been observed in disintegrated wool [15] and in longitudinal sections of follicles.

These whorl formations sometimes reduce to rather regular spirals, but more often the geometry is less ideal and adjacent fibrils fuse extensively. The drawings in Figure 5 show some of the characteristics observed. One is reminded of the spiral structures observed on the surfaces of crystals, which are attributed to growth patterns centered around dislocations in the crystal lattice. Possibly the origin of spirals and whorls will be found in similar defects in the packing of long filaments. The filaments may at first pack in a quasi-hexagonal pattern, as in the tactoids of tobacco mosaic virus. However, this regularity could scarcely persist for long. The filaments, one supposes, are not perfectly rigid, and slight defects would be introduced. In later stages it would seem more likely that further filaments (and the adhering cement) would be added as suggested in Figure 5b, which would lead to the growth of spirals of one or more arms.

A further possibility, not easy to test, is that the adhesion among the individual filaments is greater in the tangential direction than in the radial. This possibility is suggested by the results of Jeffreys, Sikorski, and Woods [9], who find that certain disintegrative procedures break the keratin into sheets rather than into bundles of filaments. Disintegration could produce either fibrous bundles or sheets, resulting from an unrolling of the "scrolls" (Figure 5c), according to the treatment.

Summary

1. The application of the technique of cutting thin sections for electron microscopy to the problem of the structure of hair and wool confirms the earlier views obtained by disintegration methods as summarized in the Review by Lindberg et al. [10]

2 Both cortex and cuticle are cellular and the normal constituents of biological cells are still present in a dried and altered form, along with the intracellular deposits of keratin in the final hair.

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Fig. 6. Cross section of the cortex prior to the final condensation, showing the spiral arrangement of the filaments within the fibrils A similar appearance is produced in the final hair if it is reduced and stained with the osmium fixative. C is the edge of the cuticle; P is a pigment granule.

and rats and these are the species which one would recommend for testing suspected carcinogens. Mice, hamsters and rats have advantages in that they are small and so easily housed and fed, and develop cancer more quickly than other species. It is generally assumed that the latent period of carcinogenesis in different species is proportional to the usual life span of the species ; the carcinogenic process would seem to be some thirty times more rapid in mice than it is in men.

Many pure lines of mice are available which might be more suitable for carcinogenicity testing than stock mice. Such mice of inbred strains have the advantage that the incidence of spontaneous tumors in the strain is known and reasonably constant. To my mind inbred animals are unsuitable for testing new substances for carcinogenic action because the strain chosen might be particularly resistant to the carcinogenic action of a particular compound. By using stock mice in which there is some genetic variation the chance that all the members of the group have such resistance is reduced. For quantitative assay of carcinogenic activity of substances which are closely related in chemical properties, pure line animals might have advantages (cf. BOYLAND and WARREN 1937, SHIMKIN and ANDERVONT 1940). If pure line animals are used for carcinogenic tests with new compounds then more than one strain should be used. First generation (F₁) hybrids have many advantages for carcinogenicity tests.

The Influence of Sex.

The incidence of induced tumors is frequently different in the sexes. Thus female mice of strains A, C3H, C and C57 black strains are more susceptible to induced hepatomata than are male animals of the same strains. Male mice of strain A are more susceptible to the carcinogenic action of small doses of 3 : 4 benzpyrene than are female mice. Because of this sexual difference in response to carcinogenic stimuli new agents should be tested on groups of each sex.

Route of administration.

In addition to skin painting — the method of application in the first successful experiments in carcinogenesis — materials may be administered by inhalation, oral administration (either in food or by instillation into the stomach), or injection by intradermal, intramuscular, intraperitoneal or intravenous routes. Each of these methods of administration has advantages in different cases.

Thus, skin painting is particularly suitable for testing products such as oil and coal tar derivatives which might induce skin cancer in man. Skin tumors can be observed as they appear which facilitates this type of experiment. Most of the investigations on co-carcinogenesis have been carried out by skin paintings on mice and such investigations allow one to determine of a substances has either initiating or promoting (i.e. co-carcinogenic) action. The possible importance of co-carcinogenesis is discussed later.

Inhalation and nasal insufflation are methods of application of materials which have in general not been effective in the past but are of obvious importance in view of the great increase in the incidence of lung cancer in man. One of the difficulties is that aerosols of carcinogenic compounds are dangerous to handle. Bronchial carcinomata resembling those occuring in man appear to have been produced with aerosols of beryllium (VORWALD 1952) and radioactive strontium (LISCO and FINKEL 1954).

It could be argued that the appropriate method of testing compounds which might be used in food, or drugs to be taken orally, would be by oral administration. Such substances should indeed be tested in this way but testing by parenteral injection is sometimes more effective and convenient. If the material under test is a potent carcinogen then the mixing of large amounts of food containing the material and the prevention of contamination of personnel by the food scattered by the experimental animals is difficult. The measurement of the amount of carcinogen consumed in such experiments is not easy.

The technique of injecting material into the stomach of animals with a syringe and blunt needle or plastic tube overcomes some of these difficulties, and is particularly suitable for studying substances which might induce cancer of the stomach on ingestion.

Tests of a number of the carcinogenic water soluble dyes such as Light Green FS (SCHILLER 1937) which proved to be carcinogenic following injection into rats, have formed the basis on which such dyes have been prohibited as food additives in Europe.

THE DETERMINATION
OF CARCINOGENIC ACTIVITY

BY

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The determination of carcinogenic activity presents difficult problems many of which are still unsolved and are of great interest and importance. Decisions as to whether a substance should be used in food, medicine or in other ways must depend on such determinations. Any tests for cancer producing power must necessarily take much time and material and may, in the end, be inconclusive.

Knowledge of carcinogenic activity is needed for immediate application so that the public can be protected from carcinogenic substances in the environment or in food or drugs. The study of carcinogenesis is also of interest as pure science and may lead to understanding of the underlying causes of those cancers which are now considered to be spontaneous. This report which is put forward as a basis for discussion, is mainly concerned with environmental carcinogens and the quantitative aspect of the problem is not emphasised because it is considered that even the smallest trace of a carcinogen is dangerous. The available data on the quantitative aspect of carcinogenesis by hydrocarbons do not suggest that there are safe concentrations of carcinogens.

The Choice of Species.

For most practical purposes we should like to know whether a substance will produce cancer in man. We know that cigarette smoke, chimney soot, carbon black, certain mineral oils, coal tar, 2-naphthylamine, benzidine, 4-aminodiphenyl, asbestos, sodium arsenite and stilboestrol cause cancer in man, but of these only the last two are used in medicine. The carcinogenic action of all these materials except asbestos and arsenicals has been demonstrated in one animal species or another. Sodium arsenite is particularly in-

teresting for although it undoubtedly induces cancer in man it does not produce cancer in any experimental animal in which it has been tested. A less extreme example of « species » difference is found with 2-naphthylamine which causes bladder cancer in man and in dogs but not in any other species of experimental animal.

The choice of experimental animals is limited to vertebrates because cancer, as it occurs in man, is known only in such animals. Although fish, amphibia and reptiles develop malignant tumors they have been used only occasionally in the study of carcinogenesis. Tumors have been induced in several species of birds (e.g. PEACOCK 1933, DURAN-REYNALS, SHRIGLEY and DE HOSTOS 1945) but these would seem to offer any special advantages for testing suspected carcinogenic substances. Monkeys appear to be very resistant to injected carcinogenic hydrocarbons (PFEIFFER and ALLEN 1948). Tumors have been induced in cats (HARDING 1947) guinea pigs (e.g. SHUBIK and MAGEE 1944) in cotton tail rabbits (e.g. SYVERTON, BERRY and DASCOMB 1942) and in hamsters (e.g. HALBESTAEDTER 1946) but none of these species have been used for routine testing of carcinogenic compounds.

The observation of HUEPER and WILEY (1937) that dogs develop bladder cancer on treatment with 2-naphthylamine may be of great value in studying bladder cancer, but tumors have never been induced in less than a year so that investigations with dogs are extremely costly and in testing new compounds there would seem to be advantage in using them.

The animals which have been used most frequently in experimental carcinogenesis (and also in cancer chemotherapy) are ro-

should be minimal in order to reduce damage or irritation. In mice doses of 10 ml. per Kg. body weight are convenient to give either by injection into tissues or instillation into the stomach. Doses of 5 ml. per Kg. body weight are convenient for rats.

Dose employed.

The dose employed should, in general, be the maximum tolerated, bearing in mind that cancer induction is slow so that for carcinogenesis animals must live for long periods and that the toxic action of some carcinogens is delayed and cumulative. In some cases, e.g. with 5:9:10 trimethyl 1:2 benzanthracene (BADGER et al. 1940) there is an optimum dose for carcinogenesis indicating that tests at different levels are probably desirable.

If a dose response curve can be established as in the case of some carcinogenic hydrocarbons then it is possible to calculate the dose which would be expected to produce cancer in any proportion of animals.

Duration of experiment.

In testing weak or slowly acting carcinogens the animals should be treated for some considerable period ; this should be at least twelve weeks and can be one year or the duration of life of the animals. Even if treatment is stopped after one year the control and treated animals should in general be allowed to live out their life span when treatment has finished.

Recommendations have been made that treatment should be carried out over three generations of animals. Although this would be a thorough test, in the author's opinion it would be too difficult to carry out with the large number of products which need to be tested.

Controls.

The necessity of carrying out controls on the solvents used in carcinogenesis has been mentioned. In addition to such « negative » controls, « positive » controls with a known carcinogenic agent, which is similar to the substance under test in chemical properties or other features, should be carried out. By this means the possibility of a false negative

-being due to resistance of the treated animals is reduced. Potent carcinogens such as methyl cholanthrene should not be used as control substances except perhaps in marginal doses, such as might be expected to induce cancer in half of the treated animals.

With new classes of substances to be tested it is often difficult to decide upon an appropriate substance for the positive control. If, however a tested substance gives no tumors when a weak carcinogen tested at the same time gave tumors, then the significance of the test is greater than if no positive control animals were used.

For tests to show that a substance is carcinogenic the incidence in the treated group of animals must be statistically significantly higher than in the control series. This can frequently be made with greater economy of means if a large group of control animals is used and several substances are tested during the same experiment. (See table I).

Cocarcinogenesis — initiators and promotors.

Experiments carried out on mice have shown that in skin carcinogenesis there are two distinct stages, of initiation and promotion (cf. BERENBLUM 1954). Substances might therefore profitably be tested as initiators or promotors of carcinogenesis, although substances which have been described as carcinogens generally have both types of activity. In most of the experiments in this field croton oil has been the promotor or cocarcinogen employed.

To test for cocarcinogenic activity mice are treated with a single subthreshold dose of carcinogenic hydrocarbon such as 9:10-dimethyl-1:2-benzanthracene and later with repeated doses of the cocarcinogen. The recent findings of SALAMAN and ROE (1954) that urethane (ethyl carbamate) is a pure initiator (i.e. it does not produce any skin tumors unless the skin it treated later with a cocarcinogen) indicate that urethane might be more suitable than 9:10 dimethyl-1:2-benzanthracene for use as an initiator in such tests. In assays of this kind both negative tests (animals without cocarcinogen) and positive tests (animals treated with an active croton oil preparation) should be included.

In testing for initiator activity the test substance is applied to the skin which is sub-

As parenteral injection is more exact and more easily controlled it would seem to be the method of choice in the investigation of such food additives, but TRUHAUT (1955) considers that substances of this type should be administered by mixing in the animal diet. Although such dyes may not be absorbed from the rat alimentary tract they might be absorbed either from the human alimentary tract or from an abraded or otherwise abnormal tract. Absorption might be influenced by other substances present in food.

Parenteral injection is the most widely used method of administration of carcinogens and this method has obvious advantages. Cancer can be induced by a single intramuscular or subcutaneous injection of a potent carcinogen but with many substances repeated injections are necessary. Subcutaneous injections can be given frequently and are usually made twice weekly for a period of between twelve weeks and one year.

Intravenous injection has been used extensively only with mice and rabbits. In the former species the tail vein can be used and in rabbits one of the veins in the ear. Intravenous administration of solutions or suspensions of carcinogenic hydrocarbons into mice usually induces cancer of the lung (e.g. SHIMKIN and LORENZ 1912).

The implantation of pellets containing suspected carcinogens into the bladder of rodents has been of value in the investigation of bladder cancer (MAISIN and PICARD 1924 ; BONSER, CLAYSON, JULL and PYRAH 1952 ; BOYLAND and WATSON 1956). With this method the possibilities of metabolism of the administered compound are reduced so that some evidence as to whether a substance is a carcinogen per se or only after some metabolic change can be obtained. Thus, BONSER, CLAYSON, JULL and PYRAH (1952) were able to show that 2-naphthylamine did not induce bladder cancer under such conditions but that one of its metabolites 2-amino-1-naphthol did do so. Experimental bladder implantation has not been very widely used but it appears to be extremely useful in the field of carcinogenesis.

Solvents or vehicles

Substances which are soluble in water are probably best administered in aqueous solution ; even insoluble substances such as the polycyclic hydrocarbons have induced tumors following injections as aqueous colloidal suspensions (BOYLAND and BURROWS 1935). Solutions prepared for injection should be approximately isotonic with body fluids as injection of concentrated solutions of sodium chloride (cf. TOKORO 1940), glucose (cf. NISHIYAMA 1935) or fructose (cf. TAKIZAWA 1940a) have induced sarcomata. Such tumors are probably caused by the osmotic pressure of the injected solution rather than by any specific chemical action.

The classical investigations into carcinogenicity of polycyclic hydrocarbons by KENNAWAY (1930) were carried out by painting solutions of the substances on the backs of mice. Most workers have used benzene as solvents for this kind of experiment, but acetone had also been employed. Both these solvents volatilize rapidly leaving the solute deposited on the skin.

Oil soluble substances such as the hydrocarbons have usually been injected in oil solutions. In the early experiments (e.g. BURROWS 1932) the hydrocarbons were dissolved in lard but this has a number of disadvantages. It must be warmed for injection ; it is of unknown and variable composition ; it produces a few tumors, when injected alone, and the activity of a carcinogen appears to vary with different batches of lard. The vegetable oils of arachis, olive and sesame are probably more convenient than lard but some batches of these (e.g. arachis oil), are also carcinogenic so that solvent control experiments must be performed.

Synthetic tricaprylin has some advantages over the natural oils ; it is not a mixture but a substance of known structure which being saturated is less chemically reactive than the natural oils. The results of SHIMKIN and ANDERVONT (1940b) indicate that it is the solvent of choice for carcinogenic hydrocarbons It does not seem to have been used as a solvent for carcinogenesis with aromatic amines but it should be very suitable for these.

Emulsifying agents such as polyethylene glycol esters and sorbitan esters dissolve hydrocarbons and such solutions are carcinogenic to the skin and the stomachs of mice (SETÄLÄ 1954).

The volume of solution used.

The volume of the material administered

TABLE I.

Detection of Carcinogenicity in experiments where equal numbers of experimental and control animals reach tumor-bearing age and where no tumors appear in controls.

Number of animals in EACH series	Number of tumors in experimental series	Lowest % incidence in experimental series that can be detected **
* 3	3	100 0
5	3	60.0
10	4	40.0
15	4	26.7
20	4	20.0
25	4	16 0
30	4	13.3
35	4	11 4
50	4	8.0
75	4	5 3
100	4	4.0
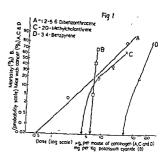
* By extrapolative technique.
** Assuming a level of significance of P = 0 025 Calculated from limits of expectation of Binomial and Poisson Distribution (Based on W. L. Stevens) Fisher and Yates 1948, Statistical Tables London, Oliver and Boyd, p 48

cinomata occurred in the mice which received the smallest doses.

Some substances which are perhaps loosely described as carcinogenic produce lesions which are not malignant. Thus urethane produces adenomata of the lung and urethane followed by application of croton oil to the skin produced skin papillomata. Such lesions are not cancers in the strict pathological sense of the term, but in making recommendations about substances of this type it must not be forgotten that adenomata or papillomata may be precancerous lesions.

The author has previously suggested that some carcinogens differ from other toxic agents in that no threshold or safe level exists and DRUCKREY (1951) has emphasized that carcinogens have cumulative and irreversible effects. Although these generalizations cannot be proved for man, they are the only reasonable assumptions on which recommendations can be made. Study of the quantitative pharmacology of carcinogenesis (BOYLAND and WARREN 1937, BRYAN and SHIM-

KIN 1940 & 1942) has shown that the induction of tumors expressed in probits varies linearly with the logarithm of the dose which in the usual form of dose-response relationship, cf. Fig. 1).

Fig 1

A - 1 2-5 6 Dibenzanthracene
C - 20 - Methylcholanthrene
D - 3 4 - Benzpyrene

[y-axis] Mortality (%) B. (probability scale) Mice with cancer (%) A, C & D
[x-axis] Dose (log scale) µg. per mouse of carcinogen (A, C and D) mg per Kg potassium cyanide

The data on 1:2-5:6-dibenzanthracene (BRYAN & SHIMKIN 1942) which agree with the findings of LETTINGA (1937) and of DOBROVOLKAIA-ZAVADSKAIA (1938) indicate that even the smallest doses of this carcinogen would induce cancer in some animals This is shown more clearly when the dose-response relationship or 1 2 5 6-dibenzanthracene is plotted linearly as it appears as a straight line passing through the origin. while methyl-cholanthrene and 3 : 4 benzpyrene have S shaped curves which are very much less steep than the curve for mortality of mice dosed with potassium cyanide. These latter curves are of the same form and indicate that there are safe doses levels for these substances administered to mice. Using the dose-response curve for 1:2-5:6-dibenzanthracene, and assuming that it remains linear even to the lowest dose, the dose which would kill a certain proportion of animals can be estimated. Extrapolation of the data of Figs 1 or 2 would indicate that a dose of 0,5 µg. of 1 2-5 6-dibenzanthracene would induce cancer in one mouse in each five hundred treated.

The data of BRYAN and SHIMKIN indicate that doses 4 µg. methyl-cholanthrene or of 30 µg. 3 : 4 benzpyrene are below the carcinogenic threshold for mice. Data on the

sequently treated repeatedly with croton oil or other effective co-carcinogen. Again both negative and positive controls (i.e. with a known initiator) must be carried out at the same time.

Pathology.

Once lesions have been obtained following treatment with a carcinogen they must be examined histologically. Many examples of non-malignant lesions such as granulomata which macroscopically resemble cancer, have been mistaken for true cancer.

Tumors must then be classified particularly into hyperplastic or metaplastic lesions, benign tumors such as adenomata and papillomata, and the malignant carcinomata and sarcomata. From the type of tumor, indications can be obtained as to the possibility of the tumor having arisen spontaneously and thus independently of the carcinogen applied.

The significance of non-malignant tumors in the practical assessment of the hazard to human health is discussed later.

Other tests.

Up to the present no short term tests have been devised which would give a valid indication as to whether a substance is carcinogenic or not. As carcinogenesis is a radiomimetic effect (cf. BOYLAND 1951) substances which produce other radiomimetic effects are likely to be carcinogenic. The most widely studied effect from this point of view is the inhibition of growth by carcinogens first observed by HADDOW (1935). The use of this test has lead to the discovery of new carcinogenic agents such as the aminostilbenes (HADDOW, HARRIS, KON and ROE 1948).

The greying of hair of colored mice following intradermal injection is another effect which parallels carcinogenesis to some extent (BOYLAND and SARGENT 1951).

Carcinogens are often mutagenic (BOYLAND 1951) and cause chromosome damage (KOLLER 1954) so that any substance with these properties should be regarded with suspicion from the point of view of carcinogenesis. None of these tests however can give more than an indication that the substance may be in fact carcinogenic. The occurrence of such effects in plant materials is, however, less signicant than when they are seen in animal tissues. Thus maleic hydrazide produces chromosome damage in plant cells but not in animal cells and is probably not carcinogenic. On the other hand urethane causes chromosome damage in animal cells but not readily in plants and induces tumors in mice and rats.

Interpretation.

Because experiments in carcinogenesis may take years to complete, they should be planned with care, bearing in mind the interpretations which might be placed on the results. Many of the substances which are of importance in regard to health are weak carcinogens. They may be designated as weak either because they produce cancer in a small proportion of animals or because they are slow in acting. These two aspects — the proportion of animals affected and the time taken to produce the effect — should be considered separately. If the substance produces tumors in only a small proportion of treated animals, then relatively large groups of animals must be used in both the control groups and in the treated group. If the carcinogen is slow acting, then groups of animals must be large enough for enough to survive until the time of tumor incidence to give a significant result. The numbers of animals necessary to obtain statistically significant results with values for the incidence of cancers, and assuming no tumors occur in the negative control group are indicated by date given in *Table 1.* These numbers refer to the animals surviving to tumor age. If a substance produces cancer in a number of animals which is just too small to be of statistical significance, the tests should be repeated.

A difficulty arises if the tumors which develop are of different types. For example, if in a group of twenty treated mice one lung carcinoma, one hepatoma, one sarcoma at the site of injection and one carcinoma of the kidney develop, the possibility that these tumors are spontaneous would appear to be greater than if all the tumors produced were of the same type. The data presented by LETTINGA (1937) are interesting in this respect as the large doses of 1:2-5:6 dibenzanthracene induced sarcomata, intermediate doses induced lymphosarcoma while mammary car-

The dose employed should be the maximal which the animals are likely to tolerate with repeated doses over the treatment period which should be of the order of one year. Similar groups of control animals should be dosed with the solvent employed and kept under the same conditions. If the material is injected in aqueous media the solutions should be approximately isotonic. Other groups of animals should be treated with a known carcinogen related, if possible, to the substance under test. The carcinogen used as positive control should be such as would be expected to produce tumors in about one half of the animals.

If a significant proportion of the treated animals develop cancer, then the result is clear cut but occasionally tumors develop in a number of animals fewer than required for the usual statistical significance. In the latter case the decision must be made as to whether the test shall be repeated or the substance considered to be harmless.

Another problem arises in deciding whether a compound found to be carcinogenic by injection should be prohibited from use in food or whether tests in which the compound is administered orally should be performed. In the author's opinion the use of a substance known to be carcinogenic by injection should be prohibited from general use in food, drink, cosmetics, contraceptives, detergents or other products used by the general public. If the substance is to be used in medicine, it should be prescribed only by a doctor who is in a position to assess the relative advantages and dangers which the use of the substance involves.

REFERENCES

BADGER G. M., COOK J. W., HEWETT, C. L. KENNAWAY, E. L., KENNAWAY N. M., MARTIN R. H. and ROBINSON A. M. : *Proc. Roy. Soc.* B. *129*, 439, 1940.

BERENBLUM I. : *Adv. Cancer Res.*, *2*, 129, 1954.

BONSER G., CLAYSON D. B., JULL J. W. and PYRAH L. N. *Brit. Jour. Canc.*, 1952.

BOYLAND E. : *Zweites Freiburger Symposium, Grundlagen & Praxie chemischer Tumorbehandlung*, p. 53, 1953.

BOYLAND E. : *Pharmacol. Rev.*, *6*, 345, 1954.

BOYLAND E. and BURROWS H. : *J. Path. Bact.*, *41*, 231, 1935.

BOYLAND E. and SARGENT S. : *Brit. J. Cancer*, *5*, 433, 1951.

BOYLAND E. and WARREN F. L. : *J. Path. Bact.*, *45*, 171, 1937.

BOYLAND E. and WATSON G. : *Nature*, *177*, 837, 1956.

BRYAN W. R. and SHIMKIN M. B. : *J. Nat. Cancer Inst.*, *1*, 807, 1940.

BURROWS H., HIEGER I. and KENNAWAY E. L. : *Am. J. Cancer*, *16*, 57, 1932.

DOBROVOLSKAIA-ZAVADSKAIA N. : *Compt Rend. Soc. Biol.*, *120*, 1055, 1938.

DRUCKREY H. : *Arzneimittel Forsch.*, *1*, 383, 1951.

DURAN-REYNALS F., SHRIGLEY E. W. & DE HOSKOS E. : *Canc. Res.*, *5*, 11, 1945.

HADDOW A. : *Nature*, *136*, 868, 1935.

HADDOW A., HARRIS R. J. C., KON G. A. R. & REE E. M. F. : *Phil. Trans. Roy. Soc.*, A. *211*, 147, 1948.

HALBERSTAETER L. : *Am. J. Cancer*, *39*, 351, 1940.

HARDING H. E. : *Ann. Report. Brit. Emp. Cancer Camp.*, *24*, 116, 1947.

HUEPER W. E. & WOLFE H D. : *Am. J. Pathol.*, *13*, 656, 1937.

KENNAWAY E. L. : *Biochem. J.*, *24*, 497, 1930.

KOLLER P. : *Progress in Biophysics*, *4*, 195, 1954

LETTINGA T. W. : *De Carcinogene Werking van Kleine doses 1:2:5:6-dibenzanthracene. Van Goram & Co, Assen*, 1937.

LISCO H. & FINKEL M. : *Fed. Proc.*, *8*, 360, 1954

MAISIN J. & PICARD E. : *Comp. Rend. Soc. Biol*, *91*, 799, 1924.

NISHIYAMA Y. : *Gann*, *29*, 1, 1935.

PEACOCK P. R. : *Proc. Roy. Soc.*, B. *113*, 285, 1933.

PFEIFFER C. A. & ALLEN E. : *Cancer Res.*, *5*, 589, 1945.

SALAMAN M. H. and ROE F. J. C. : *Brit. J. Cancer*, *7*, 472, 1953.

SCHILLER W. : *Am. J. Cancer*, *31*, 486, 1937.

SETÄLÄ K. : *Science*, *120*, 1075, 1954.

SHIMKIN M. B. and ANDERVONT H. B. : *J. Nat. Cancer Inst.*, *1*, 57, 1940.

SHIMKIN M. B. and ANDERVONT H. B. : *U. S. Public Health Rep.*, *55*, 537, 1940.

SHIMKIN M. B. and LORENZ E. : *J. Nat. Cancer Inst.*, *2*, 499, 1942.
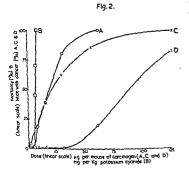
SHIMKIN M. B. and MIDER G. B. : *J. Nat. Cancer Inst.*, *1*, 707, 1941.

SYVERTON J. T., BERRY J. B. and DASCOMB H. E. : *Cancer Res.*, *2*, 436, 1942.

TAKIZAWA N. : *Gann*, *34*, 1, 1940.

TOKORO Y. : *Gann*, *34*, 149, 1940.

TRUHAUT R. : *Ann. Nutr. aliment.*, *9*, 5, 1955.

VORWALD A. J. : *7th. Saranac. Symp. Scincinae New York*, 1952.

Fig. 2.

there are approximately 2000 times as many cells in a man as in a mouse, then each individual human cell would have only 1/2000 chance of being affected by the same dose of a carcinogen as a cell of a mouse given the same dose. As there are 2000 times as many cells in a man as in a mouse the final probability of any one cell being affected will be the same. This would mean that the same dose of carcinogen would be as effective in a man as in a mouse. This is similar to infection where a single cell of a pathogen or a small amount of virus might lead to the death of a large or small animal. In the case of infection the invading pathogen multiplies in the host ; in the case of cancer the original cancer cell or group of cells proliferate, but the original cancer is independent of the size of the host.

toxicity of potassium cyanide indicate that doses of less than 5 mg. per Kg. do not kill mice. On the other hand the results with 1 :2-5.6-dibenzanthracene give no indication of a non-carcinogenic dose and if such exists it must below 2 µg. as this dose induced sarcomata in two out of seventy-nine mice which were treated. If there is no safe dose for this carcinogen, which is one of the few which have been investigated quantitatively, then for prudence one must assume that there is no safe dose for carcinogens in general.

It has also been suggested (BOYLAND 1953) that the usual relationships of the effective doses of drugs being proportional to the body weight or surface area of the animal do not hold with carcinogens because cancer can develop from a few cells or possibly from a single cell. In the first place the carcinogen may be localized, in which case it would affect the same number of cells were it localized in a large or small animal. If cancer arises from a single cell and assuming equal susceptibility for the cells of the different species, the probability of cancer being produced should be independent of the size of the animal. Even if the carcinogen is not localized the effect may be independent of animal size. The chance of cancer developing is presumably dependent on the number of « effective collisions » between carcinogen molecules and susceptible cells, and there is no reason why the number of such collisions should be dependent on the number of cells exposed. If

CONCLUSIONS

As a practical measure a substance to be tested for carcinogenic action should be injected twice weekly into groups as indicated in table II.

TABLE II

Numbers of groups of animals required for assessment of carcinogenicity

	Type of animals used		
	Stock	Pure Line	F₁ Hybrids of pure lines
Number of lines	1	3	2
Sexes	2	2	2
Routes of administration	2	2	2
Dose levels	2	2	2
Species	2	2	2
Total number of animals assuming groups of 20.	320	640(¹)	480(²)

(1) Assuming that 3 pure lines of one species (e.g. mice) and stock animals of the second species (e.g. rats) are used.

(2) Assuming that 2 F₁ hybrids of pure lines of one species (e.g. mice) and stock animals of the second species (e.g. rats) are used.

in order to maintain constant pH. Table I shows the effect of various buffer concentrations on the pH of five typical urine samples. The system previously used (b) does not always bring the pH of the urine mixture within the required limits of 4·3–4·8 (Boyland, Wallace and Williams, 1955) but, by using 0·2 M acetate buffer (c) the required conditions can be established. Under these conditions neither the original alkaline buffer (d) nor a buffer of twice its strength (e) is sufficiently strong to give a final pH within the range 10·0–10·5. The system (g) was finally selected as the one giving the most constant pH conditions.

TABLE I.—*The Effect of Buffer Concentration on the pH of the Test Solution*

| | | (pH) | | | |
Specimen No.	1	2	3	4	5
	6·30	5·20	5·70	7·00	5·95
	4·85	4·65	5·00	5·25	4·85
	4·60	4·55	4·75	4·80	4·6·5
+ 0·4 M glycine buffer	9·60	8·70	8·40	9·00	8·90
+ 0·8 M glycine buffer	10·0	9·70	9·50	9·75	9·70
f. Urine + substrate + 0·2 M acetate buffer + 10% Na₂CO₃,10H₂O	10·1	10·0	9·7	10·0	9·95
g. Urine + substrate — 0·2 M acetate buffer + 10% Na₂CO₃ (anhyd.)	10·3	10·30	10·10	10·30	10·25

The stability of the phenolphthalein colour at pH values above 9·5 has been investigated as this colour tends to fade at high pH. The colour produced by a series of standard enzyme solutions was investigated at pH values between 9·5 and 11·5 and found to be maximal between pH 10·2 and 10·6. The phenolphthalein colour is stable for 2 hours within the pH range 10–10·5 so the system (g) (Table I) is satisfactory and has been used throughout this work.

Urine (1 ml.) acetate buffer (1 ml.) (0·2 M, pH 4·5) and substrate solution (1 ml. 0·05 per cent) were incubated in stoppered tubes for 18 hours at 37° in a water bath, urine (1 ml.) was added to the blank and sodium carbonate solution (1 ml. 10 per cent Na₂CO₃) was added to each. The tubes were centrifuged and the duplicates read against the blank on a Unicam S.P. 500 spectrophotometer at 550 mμ The activity was expressed in units, 1 unit liberating 1 μg. of phenolphthalein per hour at 37°.

Owing to the modification of the method of estimation, the units are different from those used previously (Boyland, Wallace and Williams, 1955), so that the normal range (Table II) is now taken as 0·05–1·2 units/ml. of urine. Values ·in the pH range of 5·0–7·0 are regarded as normal and deviation from this range is an indication of infection of the urine. The specific gravity of the urine provides a check on the complete collection of the specimen so that both pH and specific gravity have been quoted for each specimen.

It has been suggested (Boyland et al., 1955; Boyland and Williams, 1956) that both the metabolism of tryptophan and the β-glucuronidase activity of the urine play a part in the production or maintenance of bladder tumours The amount of tryptophan in the diet might affect the apparent urinary β-glucuronidase activity, because tryptophan metabolites conjugated with glucuronic acid might act as competitive inhibitors. A number of specimens have been examined after dosing the patients with 2 g. of L-tryptophan. Such specimens are indicated in the tables by the presence of the letter T after the figure for urine volume. All specific gravities are given in direct urinometer readings, thus a figure of 10 indicates a specific gravity of 1·010.

ENZYME ACTIVITY IN RELATION TO CANCER

THE URINARY β-GLUCURONIDASE ACTIVITY OF PATIENTS SUFFERING FROM MALIGNANT DISEASE

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A CONNECTION between the process of cell proliferation and the increase in tissue β-glucuronidase has been suggested by Kerr and Levvy (1947) and Levvy, Kerr and Campbell (1948). Fishman (1947) and McDonald and Odell (1947 ; Odell and McDonald, 1948) have investigated the changes of serum β-glucuronidase activity in human pregnancy and Fishman (1947), Fishman and Anlyan (1947a, 1947b) and Fishman, Anlyan and Gordon (1947) have shown that β-glucuronidase is present in enhanced amounts in human and animal cancer tissues.

The β-glucuronidase activity of body fluids has been investigated by several workers in the hope of developing a method of diagnosis or prognosis of cancer. The β-glucuronidase activity of blood serum, ascitic and pleural fluids (Fishman, Mark   950), spinal and ventricular fluid (Anlyan and   Fishman, Kasdon and Homburger, 1950) has   from various forms of cancer especially cancer of the breast and cervix.

Boyland, Wallace and Williams, (1955a, 1955b) have shown that cancer of the bladder is associated with high β-glucuronidase levels in the urine, serum and bladder tissue. The present work deals with the urinary β-glucuronidase values of cases of cancer of sites other than the bladder. All the patients were in hospital, on balanced diets, and all the specimens were collected before any operative procedure was undertaken.

EXPERIMENTAL

The urine was collected in 2-litre vessels containing thymol dissolved in benzene (10 ml. of 20 per cent solution). Individual 24-hour specimens were measured and their pH and specific gravity determined. Samples were centrifuged at 450 g. for 15 minutes and the supernatant examined generally within 24 hours of collection being completed.

The estimation of β-glucuronidase activity in urine

The method used was essentially that of Talalay, Fishman and Huggins (1946) which has been modified for routine analysis (Boyland, Wallace and Williams, 1955). The method has been further modified by increasing the strength of the acetate buffer (pH 4·5) since, especially in the case of grossly infected urine, the urine is sometimes alkaline and therefore greater buffering power is necessary

TABLE IV.—*Patients Suffering from Argentaffinoma*

Case No.	Sex.	Age	Urine Vol (ml.)	pH	S G.	β-glucuronidase Units/ml.
1	M.	—	1800	6·4	—	0·7
2	M.	54	1900	7·9	—	0 8
3	M.	—	—	7·9	—	0 9
4	M.	70	2020	5·6	11	1·1
			1590	5·8	12	0·8
			2320T	5·8	09	0 9
			1700T	-5·5	11	0·7
5	M.	65	735	6·4	—	1·0
6	M.	39	1025	7·3	—	3 2

TABLE V.—*Patients Suffering from Cancer of the Oesophagus*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml
1	F.	68	1890	4·8	—	0·30
2	M.	45	2050	5·8	—	0 80
3	M.	61	3320	5·8	08	1·1
5	M.	67	2220	5·8	09	1·0
			1640	5·8	08	1·4
5	M.	39	890	5 8	12	1·2
6	M.	51	1530T	5·5	12	1·3
7	M.	61	370	5·5	24	4·2
			450	5·5	26	4·2

TABLE VI.—*Patients Suffering from Cancer of the Colon or Rectum*

Case No	Sex	Age	Urine Vol. (ml)	pH	S.G.	β glucuronidase Unts/ml.
			(a) Colon			
1	M.	66	1320	5·6	—	0·27
2	F.	53	890	6·4	12	0 46
			1130	6·4	08	0·28
3	M.	47	1800	5·4	—	0·54
4	F.	58	1550	5·2	—	0·65
5	M.	68	700	5·2	09	0·77
			(b) Rectum			
1	M.	55	1250	6·0	08	1·1
2	M.	72	1500	5·5	10	1·1
			1800	5·6	10	1·2
3	M.	65	1640	6·0	15	1·5
			1310	7·0	12	1·4
4	M.	45	2100	6·1	15	1·3
			1340	6·4	20	1·7
			2370	5·5	06	1·0
5	M.	72	1450	5 5	10	1·6

Patients suffering from cancer of the larynx

Of the 20 patients suffering with cancer of the vocal cord (Table VII) only 4 have β-glucuronidase activities within the normal range, with a mean 1·7 units. Of 11 patients suffering from cancer of the larynx but not cancer of the vocal cord (Table VIII), 5 had values above the range of healthy subjects, the mean value being 1·6 units.

TABLE II.—*Normal Subjects*

Case No.		Sex		Age		Urine Vol. (ml)		pH		S.G.		β-glucuronidase Units/ml.
1	.	M.	.	30	.	3250	.	6·5	.	—	.	0·05
2	.	F.	.	—	.	1890	.	6·1	.	11	.	0·14
3	.	M.	.	29	.	3500T	.	6·5	.	—	.	0·17
4	.	F.	.	63	.	1300	.	7·0	.	—	.	0 32
5	.	M.	.	68	.	1300	.	5·8	.	—	.	0·35
6	.	M.	.	26	.	2560	.	6·4	.	12	.	0·36
7	.	M.	.	72	.	1910	.	6·1	.	10	.	0·36
8	.	F.	.	—	.	2770	.	7·0	.	22	.	0 44
9	.	M.	.	48	.	2370T	.	6·2	.	06	.	0·59
10	.	M.	.	68	.	1450	.	5 5	.	—	.	0·60
11	.	M.	.	65	.	1670	.	7 0	.	06	.	0·60
12	.	M.	.	58	.	1440	.	5·5	.	14	.	0·74
13	.	M.	.	31	.	1550	.	6·5	.	—	.	0 80
14	.	F.	.	21	.	1150	.	7 0	.	15	.	0·81
15	.	M.	.	59	.	1830	.	6 4	.	08	.	0·87
16	.	M.	.	50	.	5500	.	5·3	.	12	.	0 89
17	.	M.	.	51	.	1830T	.	6·8	.	20	.	0 90
18	.	F.	.	—	.	1100T	.	7 0	.	15	.	0·93
19	.	M.	.	71	.	2520	.	5·6	.	10	.	1·0
20	.	M.	.	31	.	1800T	.	6 3	.	—	.	1·1
21	.	M.	.	60	.	2120T	.	5 8	.	09	.	1·2

Patients suffering from cancer of the alimentary tract

The urinary β-glucuronidase of patients suffering from cancer of the alimentary tract varies with the site of the tumour. The β-glucuronidase values of 5 patients out of 8 suffering from cancer of the stomach lie within the normal range and 3 are high (Table III). The mean value is 1·2 units which is the upper figure of the normal range. Of 6 cases of argentaffinoma (Table IV) only one has an abnormally high activity and the average activity is 1·2. The β-glucuronidase actitity of 5 out of 7 patients with cancer of the oesophagus (Table V) have values within the normal limits but the average enzyme value is 1·5 units. All 5 of the cases of cancer of the colon (Table VI*a*) have low β-glucuronidase activity with an average activity of 0·52 units and the urine volumes in these cases are also low. The activities in the urines of five patients with cancer of the rectum (Table VI*b*) are on or above the normal limit with an average value of 1·4 units.

TABLE III.—*Patients Suffering From Cancer of the Stomach*

Case No.		Sex		Age		Urine Vol. (ml.)		pH		S.G.		β glucuronidase Units/ml
1	.	M.	.	48	.	1560	.	5·7	.	10	.	0 45
2	.	M.	.	42	.	2340	.	6·1	.	03	.	0 70
						2450T		6 4	.	04	.	0 50
3	.	M.	.	—	.	1100	.	7·0	.	20	.	0 60
4	.	M	.	56	.	1080	.	5 8	.	—	.	0 84
5	.	M.	.	36	.	1470	.	5·8	.	12	.	1·3
						1930		6 7	.	12	.	1 6
						1600		6 0		09	.	1·1
6	.	M.	.	39	.	900	.	5 6	.	—	.	1 5
7	.	M.	.	65	.	1240	.	5 5	.	15	.	1 7
						1120		5 6	.	12	.	1 8
8	.	M.	.	54	.	1460	.	6 2	.	19	.	2 8
						1290		6 2	.	14	.	2 9
						2040		6 8	.	10	.	2·3

Patients suffering from cancer of the bronchus

Of 11 patients suffering from cancer of the bronchus (Table IX) 5 cases have a β-glucuronidase activity within the normal range and the mean is 1·8 units.

TABLE IX.—*Patients Suffering from Cancer of the Bronchus*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	F.	72	1760	6·0	10	0·50
			1090	5·5	14	0·90
2	M.	49	2270	6·1	16	0·60
			2400T	7·0	06	1·1
3	M.	75	500	—	—	1·0
4	M.	57	1720	5·5	12	1·0
			800	5·8	13	1·2
5	M.	62	720	6·8	12	0·8
			1200	6·0	—	1·5
6	M.	—	980	6·1	10	1·6
7	M.	66	1230	6·4	21	1·7
			1980	6·4	21	1·8
8	M.	62	720	6·8	12	0·8
			1200	6·0	—	1·5
9	M.	69	1400T	6·4	15	2·4
10	M.	63	1400	7·0	14	2·4
11	M.	56	870	6·8	14	4·8
			1370	6·8	14	4·9

Patients suffering from malignant blood diseases

The urinary β-glucuronidase activity of 8 out of 10 patients suffering from leukaemia (Table Xa) was above the normal range with an average activity of 1·8 units. Cases 9 and 10 had pyrexia at the time of collection. Of 5 patients suffering from Hodgkin's disease (Table Xb) 4 had activity above the normal range and the average value is 2·5 units.

TABLE X.—*Patients Suffering from Malignant Blood Diseases*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
			(a) Leukaemia			
1	M.	44	1500	6·0	—	1·5
2	M.	24	2200	5·7	—	1·2
3	M.	50	2500	6·7	09	1·2
			2100	6·7	11	0·98
			2500	5·8	67	1·5
4	F.	71	1850	7·0	10	1·2
			1860T	7·0	09	1·2
5	F.	76	1760	6·2	—	1·5
6	F.	44	1800	6·0	—	1·5
7	M.	70	1440	6·7	12	1·9
8	F.	16	1630	7·0	20	2·7
			1410T	7·4	21	2·4
9	F.	26	1680	5·8	09	3·0
10	M	38	2020	7·0	10	3·4
			(b) Hodgkins Disease			
1	M.	34	1890	6·4	07	0·50
			1890	6·0	08	0·70
2	M.	48	720	5·8	20	1·3
			1630	6·4	15	2·7
3	M.	42	2800	5·8	05	3·0
			1800	6·1	12	2·8
			1200	6·0	10	2·7
4	F.	50	420T	5·8	28	3·2
5	M.	63	1440	5·5	20	3·6
			710	7·0	—	3·8

TABLE VII.—*Patients Suffering from Cancer of the Larynx*

(a) Cancer of the Vocal Cord

Case No.	Sex	Age	Urine Vol (ml.)	pH	S.G.	β-glucuronidase units/mL
1	M.	50	2750	5·5	10	0·60
2	M.	52	1210T	5·5	10	0. 60
			1100	5·5	08	0·70
3	M.	84	2120	5·5	11	1·2
			2290	5·5	12	1·0
4	M.	68	2630	6 7	0·4	0·8
			2380T	6·8	0·5	1·6
5	M.	63	1330	6·0	14	1·5
			2590T	7·2	07	1·0
6	M.	61	2300	6·7	10	1·5
			1490T	5·5	10	1·0
7	M.	60	1010	—	—	1·4
8	F.	—	650	5·4	—	1·5
9	M.	58	1770	5·8	21	1·5
			2060	5 9	08	1·5
10	M.	67	710	7·0	18	1·6
			1330T	6·4	10	1·5
11	M.	35	1700	5 5	19	1·7
			1010T	5·6	12	1·3
12	M.	67	1130	5·5	14	1·7
13	M.	60	710	6 0	29	1·5
			540T	5·5	16	2·3
14	M.	64	1080T	5·8	17	1·8
15	M.	61	2100	6·1	11	1·7
			2320T	5·5	10	1·6
			660	5·8	15	2·5
16	M.	38	910T	5·8	15	2·4
			710	7·0	18	1 6
17	F.	61	2500	5 8	—	2 9
			1600	6·4	11	1·1
18	M.	55	740	5·6	23	2·5
			850T	5·5	28	3·1
19	M.	83	1270	7·5	10	3·4
			1260	.7 3	13	3 7
20	M.	73	890	6·1	18	4·1
			900T	5 6	14	3·2

TABLE VIII.—*Patients Suffering from Cancer of the Larynx*

(b) Cancer of Sites other than Vocal Cord

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml
1	M.	69	2350T	5·5	10	0·6
2	M.	61	2340T	6 1	06	0 7
3	M.	57	2660	7 0	06	1 0
			2520T	7·0	14	0·7
4	M.	56	2550T	6 7	10	1·0
5	F.	84	940	6 0	10	1·0
			1530T	5 8	05	1·2
6	M.	39	890	5 8	12	1·2
7	M.	51	1530T	5 5	12	1·3
8	M.	64	2590T	6 4	09	1·6
9	M.	62	1720T	5·8	07	1·7
10	M.	54	1370T	6 9	18	2 5
11	M.	57	860T	7·0	15	2·7

Patients suffering from cancer of the prostate

(a) *Untreated patients.*—Of 14 patients with cancer of the prostate examined before treatment (Table XIII) one-half have values above normal range. The average β-glucuronidase activity is 1·5 units/ml.

TABLE XIII.—*Patients Suffering from Cancer of the Prostate*

(a) *Untreated Patients*

Case No.	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml
1	75	1700	6·0	—	0·55
2	67	1700	5·5	12	0·79
3	51	1850	6·1	08	0·95
4	62	1250	5·6	—	1·0
5	58	1920	6·5	12	1·0
6	79	1060	5·2	17	1·3
		2000	5·2	08	1·0
		1500	5·2	12	1·2
7	66	1100	—	—	1·1
8	68	1720	5·8	10	0·5
		1490	6·0	08	1·0
		1100	5·8	10	1·3
		1170T	6·7	15	2·7
9	70	1910	5·7	13	1·2
		1995	5·8	14	1·3
10	57	2600	—	—	1·4
11	63	620	5·5	20	2·2
		620T	5·5	22	1·3
12	49	1290	7·0	10	2·3
		1440T	7·1	11	1·9
13	70	1230	5·5	12	2·9
		1640	5·5	09	2·6
		1940	5·8	10	3·8
14	66	1140	7·0	06	2·9

(b) *Patients treated with stilboestrol for more than two years.*—The mean β-glucuronidase activity of the urine of a group of 7 patients receiving treatment with stilboestrol for more than two years (Table XIV) is 2·3 units and all were above the normal range.

TABLE XIV.—*Patients Suffering from Cancer of the Prostate*

(b) *Patients Treated with Stilboestrol for more than Two Years*

Case No.	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml
1	70	2070	5·5	13	1·6
		2170	7·0	13	1·5
2	69	1100	6·1	21	1·8
		1930	5·7	11	1·8
3	73	1590	6·0	20	2·0
			6·9	13	1·5
4	66	970	5·8	15	2·2
		1020	5·8	13	2·0
5	72	2580	2·3	12	2·6
		1570	6·4	20	
6	68	1360	7·0	15	2·9
		1020	7·0	19	3·2
7	69	1650	7·0	17	2·9
		1400	7·0	15	3·3

Patients suffering from cancer of the breast

Of 15 patients suffering from cancer of the breast (Table XI), 8 are above the normal range of activity. Case 5 is a male with cancer of the breast. The average β-glucuronidase activity was 1·5 units.

TABLE XI.—*Patients Suffering from Cancer of the Breast*

Case No.	Age	Urine Vol. (ml)	pH	S.G.	β-glucuronidase Units/ml.
1	55	1250	6·7	09	0·3
2	52	2500	5·8	—	0·8
3	47	1820	5·5	—	1·0
4	48	2400	6·9	10	1·2
5	42	1910T	5·5	12	1·2
6	55	1200	5·5	—	1·2
7	55	1750	6·2	—	1·2
8	62	1570	4·8	—	1·5
9	53	1820	7·0	08	1·6
10	61	2000	6·5	—	1·9
		1860	9·3	13	1·3
11	48	1140	6·0	18	2·2
		1800T	5·8	14	1·3
12	38	1375	6·5	—	1·8
13	78	2020	—	—	2·2
14	54	960	4·5	—	2·5
15	63	1200	5·6	14	2·6
		1630T	5·6	12	2·5

Patients suffering from cancer of the testicle

Of 15 patients suffering from cancer of the testicle (Table XII) 10 have β-glucuronidase values above the normal range. The average β-glucuronidase value is 1·5 units.

TABLE XII.—*Patients Suffering from Cancer of the Testicle*

Case No.	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	29	1800	—	—	0·50
2	39	1400	5·4	—	0·85
3	45	610	5·8	24	1·0
4	35	1750	6·5	—	1·0
5	55	1830	8·5	11	1·2
6	37	2190	7·6	14	0·8
		2000T	7·0	12	1·6
7	45	2100	6·1	15	1·3
		1310	6·4	20	1·7
		2370	5·5	06	1·0
8	18	1500	5·5	—	1·5
		900	6·0	—	1·3
9	31	2100	7·0	—	1·4
		2100	—	—	1·4
		2200	6·5	—	1·6
10	31	2100	7·0	—	1·4
11	18	1500	5·4	—	1·5
12	30	1500	5·5	—	2·5
		2550	5·8	—	1·2
13	68	1270	5·5	10	2·5
		1330	5·5	10	2·2
		1730	5·5	12	2·0
14	39	1250	5·9	18	1·8
		450T	5·5	12	4·0
15	29	1080	—	16	4·2
		940	—	18	1·7

SUMMARY

(1) The method previously used for the estimation of urinary β-glucuronidase activity has been modified so that it may be applied to alkaline or concentrated urine.

(2) Urinary β-glucuronidase activities have been investigated in patients suffering from cancer of the alimentary tract, larynx, breast, thyroid, bronchus, testicle, and prostate and also in patients suffering from leukaemia and Hodgkin's disease.

REFERENCES

ANLYAN, A. J. AND STARR, A.—(1952) *Cancer*, 5, 578.
BOYLAND, E., WALLACE, D. M. AND WILLIAMS, D. C —(1955a) *Brit. J. Cancer*, 9, 62.—(1955b) *Brit J. Urol.*, 27, 11.
Idem AND WILLIAMS, D. C.—(1956) *Biochem. J.*, 64, 578.
FISHMAN, W. H.—(1947) *Science*, 105, 646.
Idem AND ANLYAN, A. J.—(1947a) *Ibid.*, 106, 66.—(1947b) *J. biol. Chem.*, 169, 449.
        '1947d) *Cancer Res.*, 7, 808.
        (—(1950) *J. Amer. med Ass.* 143, 350
        ?EIFFER, P. H. AND HOMBURGER, F.—(1950)
    Amer. J med. Sci., 220, 68
KERR, L. M. H. AND LEVVY, G. A.—(1947) *Nature*, 160, 463.
        (1948) *Biochem. J.* 41, 462.
        ?ndocrin., 7, 535.
        Obstet. Gynec., 56, 1.
        6) *J. biol. Chem.*, 166, 757.

Patients suffering from cancer of the thyroid

The results obtained from 7 patients with cancer of the thyroid (Table XV) show only two of these patients had β-glucuronidase activities greater than the upper limit of normal and in both of these cases the patients had raised temperatures at the time of collection of the specimen. The average β-glucuronidase activity of this group was 1·1 units.

TABLE XV.—*Patients Suffering from Cancer of the Thyroid*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	F.	57	1850	5·5	—	0·35
			1800	—	—	0 36
			1900	5·8	—	0·31
2	F.	70	2500	6 0	—	0·45
			1750	6 1	—	0 58
			2300	6·0	—	0·38
3	M.	48	2120	7·0	09	0·35
			1900	6 7	11	0·60
4	F.	68	1800	5·3	—	0 50
			2050	—	—	0 52
			2010	5·6	15	0·50
5	F.	—	1500	6·1	18	0 80
			1675	6 1	22	0·50
6	F.	—	1130	6·7	19	1·8
			980	6·7	09	1 6
7	F.	68	570	7·0	15	3·4
			500	6·4	19	4·1

DISCUSSION

The β-glucuronidase excretion has been expressed as activity per unit volume of urine but in all cases the volume of the 24-hour specimens of urine have also been recorded so that total excretions of β-glucuronidase per 24 hours can be obtained. The method of estimation has been modified so that even alkaline urine specimens, previously discarded may now estimated, but in very alkaline conditions the enzyme is not stable.

The urinary β-glucuronidase activities of patients varied considerably with the site of the tumour but in no group of cases were all the results outside the normal range. This suggests that β-glucuronidase activity could not be used as a diagnostic method, although in some types of cancer such estimations might prove useful for prognostic purposes.

The average urinary β-glucuronidase activity in cancer of various sites within the alimentary tract is, in general, near the upper limit of normal but the activity in cases of cancer of the colon is low. Cancer of the thyroid also produced a decrease in urinary β-glucuronidase activity but cases of cancer of larynx,

patient's temperature, which is known to increase β-glucuronidase excretion (Boyland and Williams, unpublished), so that the increased activity may be due partly to this cause. The connection between malignant blood disease and blood glucuronidase activity and the treatment of these patients with enzyme inhibitors is being investigated.

and Williams, 1955) but urinary sulphatase hydrolyses the sulphuric esters of some carcinogenic aminophenols only slowly (Boyland, Manson, Sims and Williams, 1956) and therefore β-glucuronidase must play a part in the hydrolysis of such conjugates. If the urinary β-glucuronidase activity in patients, in which new tumours would be expected to develop, could be inhibited, then these tumours should be prevented.

The present work deals with the urinary β-glucuronidase activity of patients suffering from cancer of the bladder and the effect of treatment with β-glucuronidase inhibitors on the enzyme activity. The inhibitors used are all derived from glucosaccharic acid, which have been previously shown to inhibit β-glucuronidase *in vitro* (Karunairatnam and Levvy, 1949; Mills and Paul, 1949; and Levvy, 1952). Saccharo-1 : 4-lactone (glucosaccharo-1 : 4-lactone) is the most effective *inhibitor and the inhibitory action of saccharates used in these investigations is probably due to the 1 : 4-saccharolactone* which they contain or is produced from saccharate in the body. Saccharic acid is readily and reversibly converted to a mixture of saccharo-1 : 4-lactone and saccharo-3 : 6-lactone.

Saccharo-1 : 4-lactone (II) is formed from saccharic acid (III) by loss of the elements of water. This dehydration reaction which is reversible gives the lactone (II) which has a structural resemblance to glucosiduronic acids (*cf.* I). Glucosaccharo-1 : 4-lactone is probably an active inhibitor of β-glucuronidase because *of this similarity in structure which allows it to occupy the enzyme centre which is concerned with β-glucuronidase activity.* Glucosaccharo-3 : 6-lactone (IV) which is also formed from saccharic acid and only differs from the 1 : 4-lactone, in the position of hydroxyl groups is a poor inhibitor. (For formulae I to IV see page 585).

EXPERIMENTAL

The β-glucuronidase activity is almost always increased in the urine of patients with cancer of the bladder and remains high in most cases even after the tumour has been removed (Boyland, Wallace and Williams, 1955). The method of estimation has since been modified (Boyland *et al.,* 1957) in the following ways—(a) A 20 per cent solution of thymol in benzene is used as a preservative as it is more effective than the benzene used in the original work. (b) The buffer concentration has been increased so that the activity of alkaline urine specimens (previously discarded) may also be estimated. The enzyme is unstable in strongly alkaline solution. The upper limit of the normal range using this method is 1·2 units/ml. All β-glucuronidase values are expressed as units of activity per ml. of urine. The unit of activity is defined as being that which liberates 1 μg. of phenolphthalein per hour at 37° C. In all cases the total 24 hr. output of urine was collected and the test performed on a 1 ml. aliquot of this; the total 24 hr. volume being also quoted so that the total 24 hr. excretion may be calculated. All patients included have a history of more than 2 years of cancer.

The treatment of patients with enzyme inhibitors

Patients have been treated with a number of inhibitors (supplied by Messrs. Kemball, Bishop and Co. Ltd., London, E.3) to decrease the β-glucuronidase activity in urine. The estimation of urinary β-glucuronidase involves a 3-fold dilution so that the apparent inhibition obtained is always less than the actual

effect in the urine. Patients were treated with solutions of the inhibitors flavoured with " fruit concentrates ".

(a) *Calcium gluconate.*—The average inhibition of urinary β-glucuronidase activity on treatment with 10 g. calcium gluconate is only 15 per cent (Table I).

TABLE I.—*Patients Suffering from Cancer of the Bladder Treated with Calcium Gluconate*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol /24 hr.	Units/ml.	Vol /24 hr.	Units/ml.
1	M.	59	2750	1 8	2700	1·2
			2750	1 6	2500	0·8
2	,,	63	1400	5 8	2750	3·8
			2350	5·5	2750	5·8
			4000	5·8	—	—
3	,,	77	1800	1 7	2400	1·7
			2000	1 9	2850	1·8
			1950	2 0	2850	1·6
4	,,	67	2750	1·5	3100	1 4
			3500	1 8	2700	1·3
			2750	1 2	3700	1·4
5	,,	65	2900	1·9	3100	1·6
			3100	2 1	2500	1·3

(b) *Sodium ammonium saccharate.*—A patient with multiple tumours of the bladder was treated with sodium ammonium saccharate solution (10 g. per day for 1 week) (Fig. 2). The treatment was then stopped and the β-glucuronidase activity rose to the original value within 3 days. The variation in enzyme activity and the volume of the 24 hr. urine specimen is also recorded. The results obtained from 4 patients treated in a similar manner (Table II) indicate that the treatment reduced the β-glucuronidase activity by 31 per cent.

TABLE II.—*Patients Suffering from Cancer of the Bladder Treated with Sodium Ammonium Saccharate*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol./24 hr.	Units/ml.
1	M.	52	3700	3 2	3100	1·2
			4000	2·7	3500	1·7
			3100	2 6	3900	1 2
2	,,	75	1700	3 0	3300	2·7
			2000	2 3	2800	2·0
			1800	2·3	—	—
3	,,	62	2500	1·6	1900	1·1
			4300	1·3	2100	1·0
			2700	1·7	—	—
4	,,	38	8000	0 8	3300	1·1
			6700	1·2	3600	0 7
			9250	1·2	3500	0 9
			7300	1·0	4100	1·0

Fig. 2.—The effect of ⟨
urinary β-glucu⟨

   ○———
   ◉ ⋯

Fig. 3.—The effect of oral admin⟨
urinary β-glucuronidase act⟨

   ○———○ β-Glu⟨
   ●———● Urine ⟨

(c) *Ammonium hydrogen saccharate.*—Treatment of a patient with 10 g. per day of ammonium hydrogen saccharate (Fig. 3) produced a fall in β-glucuronidase activity of the urine. Treating 6 patients with 3·3 g. of ammonium hydrogen saccharate 3 times daily produced an average apparent inhibition of the β-glucuronidase activity of 47 per cent (Table III).

TABLE III.—*Patients Suffering from Cancer of the Bladder Treated with Ammonium Hydrogen Saccharate*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol /24 hr.	Units/ml.
1	F.	51	2700	1·4	2750	0·65
			2850	1 8	2810	0·80
			2750	1 8	2520	0·70
			3250	1·6	2750	0·65
			2520	1·7	2450	0·80
2	M.	54	3100	0·50	2800	0·30
			2850	0·65	2700	0·35
3	,,	47	3600	1·8	2000	1·2
			2500	1 4	2250	1·0
4	,,	61	1800	1 6	2100	0·60
			1650	1 7	2200	0·65
			2750	1·6	2600	0·80
5	,,	56	700	0·55	1600	1·0
			700	0·92	—	—
6	F.	56	800	2 6	2100	1·25
			2000	3 6	2600	1·1
			2000	1·9	—	—

(d) *Glucosaccharolactone.*—(1) A group of patients were treated with a boiled solution of saccharic acid which contains an equilibrium mixture of saccharic acid and 3 : 6 and 1 : 4 saccharolactones, and received 1·3 g. of saccharo-1 : 4-lactone 3 times daily. The effect of this treatment on the urinary β-glucuronidase activity (Fig. 4 and Table IV) indicates that the mean inhibition of β-glucuronidase activity is 92 per cent.

TABLE IV.—*Patients Suffering from Cancer of the Bladder Treated with Boiled Saccharic Acid Solution*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol /24 hr.	Units/ml.
1	M.	54	2000	0 90	2400	0 01
			1900	1·3	3100	0·03
			2600	1 4	3300	0 02
			1600	1·0	2900	0 00
2	F.	38	2500	1 4	2500	0 35
			2650	0·92	2610	0 21
			2520	1·6	2120	0·14
			2760	1·0	1800	0·07
3	,,	55	2550	1·6	1100	0 44
			1750	1·0	2500	0·06
4	M	48	980	3·6	1010	0 10
			910	2·7	1100	0·05
5	,,	34	3060	1·22	4050	0·08
			4460	0·96	3700	0·10

patients were given (1) no treatment, (2) treatment with pure saccharo-1 : 4-lactone (1 g. six hourly), (3) no treatment, (4) treatment with a mixture of saccharo-1 : 4- and 3 : 6-lactones/2 : 3 (2·5 g. 6 hourly), (5) no treatment. In Case 4 the urine was contaminated with blood on the seventh day which accounts for the high result obtained.
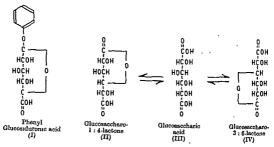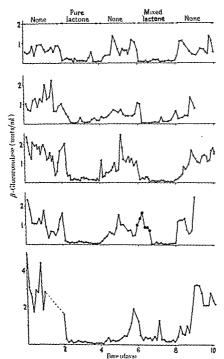
Phenyl   Glucosaccharo-   Glucosaccharic   Glucosaccharo-
Glucosiduronic acid   1 : 4-lactone   acid   3 : 6-lactone
(I)   (II)   (III)   (IV)

TABLE VI.—*Patients Suffering from Cancer of the Bladder Treated with Pure Glucosaccharo-1 : 4-lactone (1g. 4 times daily)*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml	Vol./24 hr.	Units/ml.
1	M.	53	1373	7·4	1527	0·84
			2013	5·2	1855	0·65
			1746	7·7	2120	0·51
2	,,	62	2050	7·3	2105	0·88
			1760	6·3	2110	0·22
3	F.	76	2040	10·1	1980	0·60
			1660	7·7	1565	0·90
			2125	7·1	—	—
4	M.	66	2315	5·9	2110	0·88
			2740	6·6	—	—
5	F.	—	1470	12·6	2190	0·64
			1920	11·2	1895	0·44
6	M.	61	1980	3·0	2120	0·48
			2850	2·8	1870	0·32
7	,,	82	1720	5·5	1560	0·22
			1600	4·1	1590	0·37

(4) The results obtained by treating patients with pure saccharo-1 : 4-lactone (0·5 g. 3 or 4 times daily) (Table VII) show a mean inhibition of 73 per cent and the effect on individual urine specimens of treating patients with 0·5 and 1 g. of saccharo-1 : 4-lactone 8 hourly is shown in Fig. 6.

3 : 6 mixture on the urinary β-glucuronidase of patients suffering from cancer of the bladder is shown in Fig. 5. Specimens of urine were collected from each of 5 patients at approximately 3 hourly intervals and the β-glucuronidase activity of the individual specimens estimated. In 5 successive periods each of 48 hr. the

Fig. 5.—The effect of oral administration of glucosaccharo-1 : 4-lactone and mixtures of 1 : 4 and 3 : 6-lactones on urinary β-glucuronidase activity in 5 patients with bladder cancer.

O———O β-Glucuronidase activity (units/ml.).
●———● β-Glucuronidase activity (units/ml.) in urine containing blood.

TABLE VII.—*Patients Suffering from Cancer of the Bladder Treated with Pure Glucosaccharo-1 : 4-lactone*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol /24 hr.	Units/ml
(a) Dose 4 × 0·5 g./day)—						
1	M.	52	2720	1·1	2780	0·12
			2585	0·83	2740	0·12
2	,,	63	1415	1·5	1205	0 16
			1600	4·4	—	—
3	F.	43	1100	0·53	1550	0 06
			1700	0·38	1835	0·13
(b) (Dose 3 × 0·5 g./day)—						
1	M.	43	1330	6·5	1515	3·8
			1205	6·9	1405	3·1
2	,,	55	2350	1·4	2140	0·17
			1560	1·5	2310	0·25
3	,,	68	2800	0·93	1830	0·10
			1590	1·0	2060	0·22
4	,,	71	2360	1·7	2290	0·37
			1510	1·9	2750	0·47

The results indicate that the urinary β-glucuronidase activity can be maintained at a low level by the administration of either the pure 1 : 4-lactone or the mixture of lactones. Patients are being treated with 1 g. saccharo-1 : 4-lactone *t.d.s.* for clinical trials.

DISCUSSION

The urinary β-glucuronidase activities of patients who have been suffering from cancer of the bladder for some years are almost all raised above the corresponding value for normal subjects. The increased activity does not depend upon infection although, in a few cases infected by *B. coli*, this may play some part. The enzyme is stable up to pH 9·0 and the method of estimation allows urine of pH less than this value to be used. The results are in agreement with those obtained previously (Boyland *et al.*, 1955) using an earlier technique.

The urinary β-glucuronidase activity of patients with cancer of the kidney (hypernephroma) is low compared with that of bladder cancer patients, the average value being within the normal range. Fishman and his co-workers have shown that cancer cells have a high β-glucuronidase activity but the high urinary activity in cancer of the bladder cannot be due to the urine being in contact with malignant tissue as urine from patients with cancer of the kidney should also be rich in β-glucuronidase. This evidence further supports the suggestion (Boyland *et al.*, 1955) that the increased urinary enzyme concentration is probably derived from the blood, the amount of the enzyme depending both on the blood concentration and the degree to which the enzyme passes through the kidney. The relationship between the β-glucuronidase activities of serum and urine of patients with cancer of varying sites is being investigated further.

Patients suffering from cancer of the bladder have been treated with gluco-saccharo-1 : 4-lactone (4 g. per day) which reduces the activity by 90 per cent. This inhibition of β-glucuronidase would be produced with a 5×10^{-5} M saccharo-
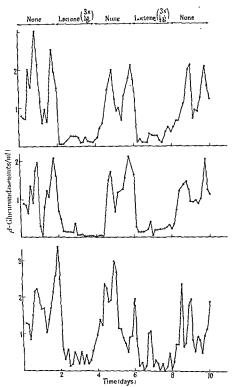
FIG. 6.—The effect of oral administration of pure glucosaccharo-1 : 4-lactone on urinary β-glucuronidase activity in 3 patients with bladder cancer.

O————O β-Glucuronidase activity (units/ml).

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REFERENCES

ALLEN, M.; BOYLAND, E., DUKES, C. E., HORNING, E. S. AND WATSON, J. G.—(1957) *Brit. J. Cancer*, 11, 212.
BONSER, G. M., BRADSHAW, L., CLAYSON, D. B. AND JULL, J. W.—(1956) *Ibid.*, 10, 539.
BOYLAND, E., GASSON, J. E. AND WILLIAMS, D. C.—(1957) *Ibid.*, 11, 120.
Idem, MANSON, D., SIMS, P. AND WILLIAMS, D. C.—(1956) *Biochem. J.*, 62, 68.
Idem, WALLACE, D. M. AND WILLIAMS, D. C.—(1955) *Brit. J. Cancer*, 9, 62.
Idem AND WILLIAMS, D. C.—(1954) *Biochem. J.*, 56, xxix.—(1956) *Ibid*, 64. 578.
HORNING, E. S.—(1956) *Brit. J. Cancer*, 10, 678.
KARUNAIRATNAM, M. C. AND LEVVY, G. A.—(1949) *Biochem. J.*, 44, 599.
LEVVY, G. A.—(1952) *Ibid.*, 52, 464.
MILLS, G. T. AND PAUL, J.—(1949) *Ibid.*, 44, xxxiv.

1 : 4-lactone solution (Levvy, 1952) which is equivalent to about 10 mg. per litre. This indicates that only 0·5 per cent of the dose administered is excreted in the urine. The greater part (99·5 per cent) of the lactone is inactivated either by metabolic processes or reaction with water or other body constituents. This treatment reduced the urinary β-glucuronidase level to values below the average normal level and by this means it is hoped to decrease the amount of free carcinogens in the urine and thus reduce the incidence of new tumours in patients, who have had tumours removed. Twenty bladder cancer patients are at present under treatment with glucosaccharo-1 : 4-lactone, but this treatment has not been used long enough to allow any conclusions to be drawn as to the clinical benefit.

The authors realise that to treat patients who have had cancer of the bladder with compounds which will reduce the concentration of carcinogenic agents in the urine, is probably too late to act too late. Because of the long lag between the application of the carcinogenic stimulus and the occurrence of the cancer, the first stimulus had probably taken effect before treatment began. This is, however, not certain and the reduction of carcinogens in the urine may reduce the chance of tumours developing. The tumours may, however, be dependent on the continued presence of the carcinogen in the way that the growth of kidney tumours, induced in hamsters with stilboestrol is dependent on the continued presence of stilboestrol (Horning, 1956). If the tumours of the bladder were dependent on the urinary carcinogen then treatment with saccharo-1 : 4-lactone could prevent growth of established tumours.

Bladder cancer caused by aromatic amines among men in the chemical industry is probably due to metabolites of the aromatic amines being hydrolysed by β-glucuronidase in the urine to give aminophenols. Stringent precautions are now taken in the chemical industry to prevent exposure to the carcinogenic amines. In such factories, however, if accidents happen the carcinogenic effect of exposure of personnel should be reduced by administration of saccharo-1 : 4-lactone immediately following the exposure and for four or five days. In such cases the treatment should prevent the liberation of the carcinogen in the urine.

SUMMARY

The *in vivo* effect of various derivatives of saccharic acid which are β-glucuronidase inhibitors has been investigated ; oral administration of 1 g. saccharo-1 : 4-lactone every 6 hours decreases the enzyme activity in the urine by 90 per cent.

Treatment with saccharolactone should therefore reduce the liberation of free carcinogenic aminophenols in urine. Such treatment is recommended for men accidentally exposed to carcinogenic aromatic amines and is being tried in prophylactic treatment of patients who have had bladder cancers removed or destroyed.

of chemically inert plastic materials observed by Oppenheimer, Oppenheimer, Danishefsky, Stout and Eirich (1955).

The drawback to this method is similar to that encountered *in investigation of* cocarcinogenesis with croton oil. Although treatment with croton oil *without* an initiator gives some tumours in mice, the *activity of an initiator* can be estimated by the increase in the tumour incidence after treatment with an *initiator.*

As already mentioned, the implantation of pellets of paraffin wax *weighing between 80 and 170 mg.* induces papillomas (Bonser *et al.*, 1953), but the implantation of *10 mg.* pellets did not do so (Rudali *et al.*, 1955). It is probable therefore that the implantation of pellets smaller than 10 mg. (*e.g.* 5 mg.) *would induce* fewer tumours in the control mice.

This technique has been used in the present communication *in order to* test the following groups of compounds :

(1) Miscellaneous compounds.

(2) 1 : 2-5 : 6-Dibenzanthracene, 1 : 2-5 : 6-dibenzanthracene-3 : 4-quinone and a metabolite 2-phenylphenanthrene-3-2'-dicarboxylic ·acid which Bhargava, Hadler and Heidelberger (1955) consider to be the active carcinogenic metabolite of the hydrocarbon.

(3) Metabolites and other derivatives of 2-naphthylamine.

(4) *ortho* Aminophenols derived from other carcinogenic aromatic amines and related compounds.

(5) Metabolites of tryptophan some of which are *ortho* aminophenols *and so* related to the metabolites of some carcinogenic aromatic amines.

In studying the metabolism of 2-naphthylamine in rats and rabbits 16 metabolites (Table I) of the amine have been detected, but free 2-amino-1-naphthol has not been found in freshly excreted urine (Boyland and Manson, 1955, 1957 ; Boyland, Manson and Orr, 1957). Five of these metabolites have been tested in the present work.

One of the metabolites is 2-amino-1-naphthol sulphuric ester which Bonser, Bradshaw, Clayson and Jull (1956) have found to be non-carcinogenic by the technique of bladder implantation. Human and animal urines contain arylsulphatases which might have been expected to liberate the carcinogenic 2-amino-1-naphthol from the sulphuric ester. An examination of the action of sulphatases on sulphuric esters of aminophenols (Boyland, Manson, Sims and Williams, 1956) however, showed that 2-amino-1-naphthol sulphuric ester and other sulphuric esters of *o*-aminophenols derived from carcinogenic amines were not hydrolysed by sulphatase. This resistance of the sulphuric ester to enzymic hydrolysis explains the lack of carcinogenicity of this metabolite.

EXPERIMENTAL

(1) Pellets weighing between 8 and 12 mg. were made up from a molten mixture of 4 parts paraffin wax (m.p. 56°, filtered, from G. T. Gurr, Ltd.) and 1 part of suspected carcinogen as described by Jull (1951).

(2) Pellets weighing between 9 and 11 mg. were prepared from a mixture of 4 parts cholesterol (Roche Products) and 1 part suspected carcinogen ground together and compressed in a tablet-making machine.

The same samples of paraffin wax and cholesterol were used throughout the experiments.

CANCER OF THE URINARY BLADDER INDUCED IN MICE WITH METABOLITES OF AROMATIC AMINES AND TRYPTOPHAN

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SENSITIVITY of the bladder epithelium to carcinogenic stimuli was demonstrated by the induction of cancer following the introduction of coal tar directly into the bladders of rats (Maisin and Picard, 1924 ; Picard, 1927). Bonser, Clayson, Jull and Pyrah (1953) showed that the implantation of relatively large paraffin wax pellets without added chemicals into the bladders of rats induced papillomas. On the other hand Rudali, Chalvet and Winternitz (1955) were unable to obtain tumours when small pellets of paraffin wax or cholesterol were inserted into the bladders of rats, but carcinomata were produced with wax or cholesterol pellets containing phenazine, 1 . 2-5 · 6-dibenzphenazine or 20-methylcholanthrene. The bladders of mice seem to be more resistant to the apparent carcinogenic action of inert solids than are those of rats so that mice are more suitable animals for the testing of bladder carcinogens.

Jull (1951) developed the technique of surgical introduction of wax pellets containing carcinogens into the bladders of mice and this has been used very effectively by Bonser, Clayson and Jull (1951) and by Bonser, Clayson, Jull and Pyrah (1952) in studying the induction of bladder cancer. These authors found that 2-naphthylamine, which induces bladder cancer in dogs and man, does not cause cancer when introduced into the bladders of mice. On the other hand pellets containing 2-amino-l-naphthol, which is a known intermediary metabolite of 2-naphthylamine, produced cancer of the bladder Thus they showed that the method could give some indication as to whether a carcinogen was acting directly or after metabolic changes. The fact that bladder tumours were obtained with 20-methylcholanthrene and 3 4-5 6-dibenzcarbazole suggests that these polycyclic compounds act without undergoing previous metabolic changes

In the present work the technique described by Jull (1951) has been modified in two ways (cf Boyland and Watson, 1956) Firstly, the substances under test are mixed with four parts of cholesterol and compressed into pellets instead of being mixed with molten wax Secondly, the opening in the bladder through which the pellet is introduced is tied off with thread instead of being sewn A disadvantage of this method is that some tumours are induced by cholesterol pellets alone and this must be considered in assessing the carcinogenicity of the substances tested Bonser, Bradshaw Clayson and Jull (1956), in a thorough investigation of this technique, obtained tumours in a proportion of mice treated with pellets of paraffin wax This induction of cancer by chemically inert material is perhaps analogous to the induction of sarcomata by subcutaneous implantation

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TABLE I—*cont.*

9. 2-Amino-6 naphthol sulphuric ester.

10. 2-Amino-6-naphthyl glucosiduronic acid.

11. 2-Acetamido-6-naphthyl glucosiduronic acid.

12. 2-Acetamido-6-naphthol sulphuric ester.

13. 2 - Acetamido - 5 : 6 - dihydroxy - 5 : 6 - dihydro-naphthalene.

14. 2 - Acetamido - 5 · 6 - dihydroxy - 5 : 6 - dihydro-naphthalene glucosiduronic acid.

15. 2 - Acetamido - 5 · 6 - dihyrdoxynaphthalene sulphuric ester.

16. 2 - Acetamido - 5 · 6 - dihydroxynaphthalene glucosiduronic acid.

TABLE I

Metabolites of 2-naphthylamine

1. 2-Amino-1-naphthol sulphuric ester.

2. 2-Acetamidonaphthalene.

3. 2-Naphthyl sulphamic acid.

4. 2-Naphthylamine-N-glucosiduronic acid.

5. 2-Amino-1-naphthol glucosiduronic acid.

6. 2-Amino-1-naphthol sulphuric ester N-gluco-siduronic acid.

7. 2-Amino-6-naphthol

8. 2-Acetamido-6-naphthol
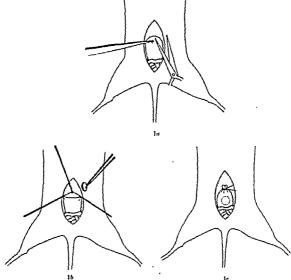
Fio. 1.—Stages in the process of introducing a pellet into the urinary
bladder of a mouse.   (Diagrammatic.)

or "adenomas" provided there was no invasive infiltration, or cytological evidence
of malignancy. The term "carcinoma" was only used for tumours that were
invasive or had the histological characteristics of malignancy.

RESULTS

In most of the groups, 60 to 80 per cent of the mice survived the operation
for 30 weeks (Table II). When considering the reports recorded in the table,
.. ... ·   cinomata are all classified as "tumours" and the
                              sidered from the total incidence of "tumours".
                              not recorded. Among the mice which died in less
                 no tumours were seen, but in many cases post
mortem changes were so great that a histological diagnosis could not be made.
The "activity" is therefore considered from the ratio of number of tumour-

1 : 2–5 : 6-Dibenzanthracene, 3 : 4-quinone and 2-phenylphenanthrane-3 : 2'-dicarboxylic acid were prepared as described by Bhargava, Hadler and Heidelberger (1955).

2-Amino-1-naphthol, 4-dimethylamino-3-hydroxydiphenyl, and 1-dimethylamino-2-naphthol were prepared by hydrolysis of the sulphuric esters obtained by oxidation of the parent amines with persulphate as described by Boyland and Sims, (1954). Potassium 2-naphthylsulphamate and ammonium 2-naphthylamine-N-glucosiduronate were prepared by the methods of Boyland, Manson and Orr (1957). 2-Amino1-naphthol glucosiduronic acid was prepared by the acid hydrolysis of 2-acetamido-1-naphthol glucosiduronic acid which was isolated from urine of rabbits dosed with 2-acetamido-1-naphthol (Boyland and Manson, 1957). 3-Hydroxykynurenine was synthesised according to the method of Butenandt and Hellmann (1950).

Stock mice were anaesthetised with a mixture of 90 per cent ether and 10 per cent ethanol and maintained in anaesthesia by placing the head in a tube containing a cotton wool plug moistened with the anaesthetic. The abdominal wall was wetted with ethanol and an incision made with scissors to give access to the bladder. The apex of the bladder was held in forceps and a 3 mm. incision made in the fundus with scissors (Fig. 1a). The pellet, held in straight forceps, was then inserted into the bladder while the lumen was held open with 3 pairs of forceps (Fig. 1b). The suture was then held together with forceps and ligated close to the forceps with a single fine silk ligature (Fig. 1c) The abdominal wall was closed by sewing in two layers using a figure-of-eight stitch.

The mice were returned to their cages and maintained on a mixed diet. Any mice which appeared ill were killed, other mice were killed either at 40 weeks or 52 weeks after operation. All mice were examined at post mortem in a manner previously described by Jull (1951). The bladders were distended by the injection of Bouin's solution and fixed for 24 hours before they were cut open and examined with a hand lens.

S. . . . .
fro. . . . .
in c. . . . .

1. *Inflammatory lesions.*—These were very common and consisted usually of a dense infiltration of the mucosa with inflammatory cells of the lymphoid type resulting often in a general thickening of all coats of the bladder. These were of no significance in relation to neoplastic changes

2. *Lesions due to operative trauma* —These consisted of small fibrous nodules or islands of misplaced epithelium sometimes forming small cysts Lesions associated with the sequestration of epithelium on the suture line had to be distinguished from invasive neoplasms

3 *Neoplastic lesions* Minute foci of epithelial hyperplasia were not regarded as of neoplastic significance because although some may represent the earliest presence of neoplasm others may be inflammatory in nature The only lesions recorded were larger nodules worthy of being called " tumours ", which projected from the surface or dipped into the subepithelial tissues These were classified as either (a) benign papilloma and adenoma " or (b) carcinoma The distinction between these two stages in neoplastic proliferation is not always easy because there may be no hard and fast line of distinction between them Benign tumours according to their general cellular structure were called either " papillomas "

bearing mice to the number of mice surviving for 30 weeks. The probability that this ratio differed from the incidence in the control series was calculated by the χ^2 test.

Although no tumours were seen in the 13 mice which survived for 30 weeks or more after treatment with paraffin wax pellets, one mouse killed after 52 weeks showed hyperplastic changes of the bladder epithelium. Of the 24 mice surviving 30 weeks or more after implantation with cholesterol pellets one had a carcinoma and another mouse revealed hyperplastic changes. Thus the control treatment does not produce a completely negative result. Of the 26 groups of mice treated with substances under test, 9 contained one tumour in each group and except for the 3-hydroxy-4-aminodiphenyl group in which only 10 mice survived 30 weeks the substances implanted in these groups may be considered as inactive. These include :

    1 . 2–5 : 6-Dibenzanthracene-3 : 4-quinone
    2-Phenylphenanthrene-3 : 2'-dicarboxylic acid
    Potassium 2-naphthylsulphamate
    Ammonium-2-naphthylamine-N-glucosiduronate
    2-Amino-6-naphthol hydrochloride
    2-Acetamido-6-naphthol
    1-Dimethylamino-2-naphthol,
    4-Dimethylaminoazobenzene.

Examples of the lesions produced are illustrated in Fig. 3–8.

DISCUSSION

The technique of implanting pellets into the bladders of mice can be used to indicate whether the carcinogenic activity is due to the substance itself or to a

to their biological action.

1. *Miscellaneous substances*

Maleic hydrazide was tested in the present experiments because Darlington and McLeish (1951) had found it to be a mitotic poison for plant cells. It did not induce cancer . . . . with the findings of Barnes and Haddow . . . . obtain a significant incidence of tumours in rats injected with this compound. Although it produced chromosome damage in plant cells, it does not interfere with mitosis in cells of the Walker carcinoma, when injected into tumour-bearing rats (Boyland and Koller, unpublished observations).

Pellets containing xanthine induced carcinomata. This is in agreement with earlier work (Haddow, personal communication) in which injection of xanthine induced sarcomata in rats.

Pellets containing saccharin induced a significant incidence of bladder tumours. On the other hand, in an experiment in which 20 mice were injected twice weekly with 1 g. saccharin per kg. body weight for 12 weeks, no tumours developed, although 12 mice lived for one year and 8 mice were killed 2 years after the

TABLE II.—*The Incidence of Lesions in Mice Following Implantation of Pellets into the Bladder*

	Number of mice						Ratio of "tumours" to mice surviving 30 weeks	Probability "P"—that the tumour incidence is due to chance
	At beginning of experiment	After 30 weeks	After 40 weeks	After 52 weeks	With papilloma or adenoma	With carcinoma		
Controls								
Cholesterol only	28	24	24	0	0	1	1/24	—
Paraffin Wax only	20	13	21	4	0	0	0/13	—
Miscellaneous compounds								
Xanthine	20	15	14	7	2	2	4/15	0·04
Saccharin	20	13	13	9	1	3	4/13	0·01
Maleic hydrazide	20	14	14	0	0	0	0/14	—
Hydrocarbon derivatives								
1:2:5:6-Dibenzanthracene	20	12	11	0	0	4	4/12	0·03
1:2:5:6-Dibenzanthracene-3:4-quinone	20	13	12	0	1	0	1/13	0·4
2-Phenylphenanthrene-3:2'-dicarboxylic acid	20	14	14	0	0	1	1/14	0·7
Naphthylamine derivatives								
Potassium 2-naphthyl sulphamate‡	20	15	15	6	1	0	1/16	0·7
Ammonium 2-naphthylamine-N-glucosulfamate‡	20	13	9	5	1	0	1/13	0·5
2-Amino-6-naphthol hydrochloride‡	20	13	11	4	1	0	1/13	0·5
2-Acetamido-naphthol‡	19	11	10	0	0	0	0/11	—
2-Amino-1-naphthol hydrochloride	25	11	17	0	1	0	1/15	0·5
2-Amino-1-naphthyl glucosiduronic acid	25	17	17	0	4	3	1/17	0·01
1:1-dimethylamino-2-naphthol	20	14	14	0	1	1	2/14	0·3
1:2:5:6-Dibenzphenazine	20	11	11	10	1	0	1/11	0·5
Amines and aminophenols								
4-Dimethylamino-3-hydroxydiphenyl	20	13	11	0	1	1	2/13	0·3
4-Dimethylaminoazobenzene	20	10	12	0	1	0	1/16	0·7
2:4-Diamino-3-phenylazopyridine	20	14	12	10	0	2	4/14	0·07
2-Amino-4:5-dimethylphenol	25	18	18	0	2	5	5/18	0·02
4-Hydroxyquinoline	20	10	13	0	2	3	5/10	0·01
Tryptophan derivatives								
3-Hydroxykynurenine	35	25	10	4	1	6	7/25	0·02
Kynurenic acid	20	12	11	0	0	1	1/12	0·3
*-Methoxy-4-hydroxyquinoline-2-carboxylic acid	20	19	14	0	1	3	4/17	0·08
3-Hydroxyanthranilic acid	60	40	39	24	3	8	11/40	0·02
3-Hydroxyanthranilic acid	20	12	12	5	0	1	1/22	1·0
Methyl 3-hydroxyanthranilate	20	13	13	0	0	2	2/13	0·3
2-Amino-3 hydroxy acetophenone	25	17	17	0	1	4	5/17	0·03
5 Hydroxytryptamine creatinine sulphate	20	14	14	10	0	0	1/14	0·8

‡ Pellets made with paraffin wax in place of cholesterol.

after the latter has entered susceptible cells. The simpler explanation is, however, that the carcinogenic hydrocarbons are carcinogenic *per se*. The carcinogenic action of hydrocarbons could be due to their ability to form complexes with the purines of nucleic acid and " this association may change the nucleic acid sufficiently for chromosome aberrations to result " (Boyland, 1952). The induction of tumours with the hydrocarbon is in agreement with the findings that the polycyclic compounds 20-methylcholanthrene and 3 : 4–5 : 6-dibenzcarbazole are carcinogenic in the mouse bladder (Bonser, Clayson, Jull and Pyrah, 1952).

3. *Derivatives of 2-naphthylamine*

Unlike 1 : 2–5 : 6-dibenzanthracene, some aromatic amines appear to be indirect carcinogens which must be metabolised into active forms. 2-Naphthylamine itself possesses only very slight activity as a bladder carcinogen but 2-amino-1-naphthol is active (Bonser, Bradshaw, Clayson and Jull, 1956) and of the seven derivatives examined only 2-amino-1-naphthyl glucosiduronic acid produced a significant incidence of tumours. It had been argued (Boyland and Manson, 1955 ; Boyland, 1956) that of the 16 identified metabolites of 2-naphthylamine listed in Table I only one—2-amino-1-naphthyl glucosiduronic acid—should be carcinogenic and this compound has now been shown to induce tumours. The relatively high proportion of papillomas (4 papillomas, 3 carcinomas) induced with this metabolite is remarkable. If one assumes that the papillomas would have developed into carcinomas in time then this finding suggests that the substance is slow in action.

The insignificant incidence of tumours induced with 2-amino-6-naphthol hydrochloride an 2-acetamido-6-naphthol is of interest as these are representatives of the type of oxidation product in which the oxygen has entered the benzene ring remote from the amino group. The inactivity of these substances makes it improbable that derivatives of these (e.g., Nos. 9, 10, 11 and 12 of Table I) would be carcinogenic but nevertheless they are being tested in experiments which are now in progress. The inactivity of 2-amino-6-naphthol compared with the activity of 2-amino-1-naphthol which is an *ortho* aminophenol is in agreement with the idea that the carcinogenic activity of some at least of the aromatic amines is dependent on their metabolic conversion to *ortho* aminophenols. The susceptibility of the dog and man to the carcinogenic effect of 2-naphthylamine is probably related to the relatively high proportion of 2-amino-1-naphthol formed *in vivo* by these species. In rodents, which are more resistant to 2-naphthylamine, derivatives of 2-amino-6-naphthol appear to be the predominant metabolites (Clayson, 1953). 2-Amino-1-naphthol, however, does not usually occur free in the urine but conjugated with glucuronic acid and sulphuric acid. In the presence of β-glucuronidase the glucosiduronate may be hydrolysed, liberating the active compound. In the present experiment, 2-amino-1-naphthol glucosiduronic acid produced more tumours than did the free 2-amino-1-naphthol ; possibly because the carcinogenic activity of the latter was in part masked by toxic effects When the pellet of 2-amino-1-naphthol in cholesterol was inserted into the bladder, a dose toxic to proliferating cells might have been introduced, whereas in the case of the glucuronic acid conjugate, the free carcinogen would be liberated slowly by β-glucuronidase in the urine.

Bonser *et al.* (1956) has shown that 1-amino-2-naphthol and also 2-amino-1-naphthol were carcinogenic in the bladder of mice. Our results suggest that the

Maleic hydrazide

Xanthine

Saccharin

Formulae A

commencement of the experiment. The bladders of these mice were examined *post-mortem* as in the bladder implantation experiments. In further experiments carried out with Professor P. C. Koller, the injection of 1 g. per kg. of saccharin as the sodium salt into rats bearing the Walker Carcinoma produced no apparent chromosome damage. Fitzhugh, Nelson and Frawley (1951) noticed that feeding of saccharin to rats was associated with" an increased incidence of the ordinarily uncommon condition of abdominal lymphosarcoma " but these authors did not consider that saccharin was carcinogenic. Salaman and Roe (1956) found that saccharin had some initiating action, that is, it caused skin tumours to appear in mice when its application was followed by treatment with croton oil. The induction of bladder tumours with saccharin suggests that the presence of the solid pellet in the bladder may have a promoting action and that the method of bladder implantation detects incomplete carcinogens.

2. 1 : 2-5 : 6-*Dibenzanthracene and derivatives*

Bladder tumours were produced with 1 : 2–5 :6-dibenzanthracene but the oxidation product 1 : 2–5 : 6-dibenzanthracene-3 : 4-quinone and the metabolite 2-phenylphenanthrene-3 : 2'-dicarboxylic acid gave negative results. The parent compound thus appears to initiate a neoplastic change without biochemical elaboration. If this is the case, then the conversion of 1 .2–5 : 6-dibenzanthracene into 2-phenylphenanthrene-3 : 2'-dicarboxylic acid described by Bhargava *et al.* (1955) if of the nature of a side reaction or detoxication process. The possibility remains that the phenylphenanthrene dicarboxylic acid cannot penetrate into cells, but can be produced by metabolism of 1 : 2-5 : 6-dibenzanthracene

1 . 2 : 5 : 6 Dibenzanthracene

1 : 2-5 : 6 Dibenzanthracene
3 . 4 quinone

2-Phenylphenanthrene
3 : 2'-dicarboxylic acid

Formulae B—Hydrocarbon derivatives

A number of *ortho* aminophenols are now known to induce cancer in the bladder of mice and the problem of the mechanism of action presents itself. These amino-phenols are very reactive compounds being readily oxidised and combining with many reagents. They are also chelating agents forming complexes with metals (Charles and Freiser, 1952). For this reason the active chelating agent, 8-hydroxy-quinoline or oxine, was tested and gave tumours in 6 out of 16 mice which survived 30 weeks. Thus it is possible that this compound and the *ortho* aminophenols are active by virtue of their chelating power. The activity might therefore be due to combination of the carcinogen with the desoxyribonucleic acid (DNA) of chromo-somes through the metals of the DNA forming a double chelate as a distorted DNA molecule. Other possibilities are that the chelating agent competes with the DNA molecules for metals which are probably essential for the correct functioning of DNA of chromosomes or that they break nucleoprotein molecules as observed by Kirby (1956) *in vitro*. On the other hand the fact that Bonser *et al.* (1954) found 1-methoxy-2-naphthylamine induced bladder cancer in mice is not in agreement with this hypothesis as this compound should be devoid of chelating activity.

The carcinogenic action of 8-hydroxyquinoline is of practical interest because this substance is used as a spermicidal agent in contraceptive preparations and as a preservative particularly in tobacco in Germany (Wegner, 1955). Hoch-Ligeti (1956) has described the induction of carcinoma of the vagina in rats treated intravaginally with a contraceptive cream containing 8-hydroxyquinoline

spermicidal agents are being tested for carcinogenic activity in the vagina of the mouse.

5 *Tryptophan metabolites*

The natural history of bladder cancer in men working with aromatic amines does not differ from that of bladder cancer occurring in the general population. In the case of the occupational cancer the cause would seem to be the *ortho* aminophenols liberated in the urine by the action of urinary β-glucuronidase on excreted metabolites of aromatic amines such as 2-naphthylamine and 4-amino-diphenyl. One might expect therefore that bladder cancer in the general popula-tion is due to an excreted carcinogen. In normal metabolism in man and animals tryptophan is converted to nicotinic acid by way of kynurenine, 3-hydroxy-kynurenic and 3-hydroxy anthranilic acid ; 3-hydroxykynurenine and 3-hydroxy-anthranilic acid are *ortho* aminophenols and they are often present in human urine. 2-Amino-3-hydroxyacetophenone is another *ortho* aminophenol derived from tryptophan which is sometimes present in human urine (Dalgleish, 1955) All these naturally-occurring *ortho* aminophenols induced bladder tumours in our experiments.

Although pellets of 3-hydroxyanthranilic acid in cholesterol produced tumours, pellets of the same compound in paraffin wax did not induce a significant number of tumours (1 tumour in 22 mice). The diffusion of 3-hydroxyanthranilic acid from pellets was therefore examined. The pellets were incubated in 1 ml. water at 38°. After different intervals (indicated by the points in the figure) the aqueous phase was replaced and the 3-hydroxyanthranilic acid estimated by measurement of

substitution of the amino group as in 1-dimethylamino-2-naphthol destroyed the activity. This is surprising as usually methylation of carcinogenic aromatic amines either in the amino group itself or in the positions *ortho* to the amino group enhances their carcinogenic activity.

4. Amines and aminophenols

4-Dimethylamino-3-hydroxydiphenyl, which is the *ortho* aminophenol corresponding to 4-dimethylaminodiphenyl which had been shown to be carcinogenic in rats (Miller, Miller, Sandin and Brown, 1949), gave an indefinite result (2 tumours in 13 mice).

2-Amino-4 : 5 dimethylphenole

8-Hydroxyquinoline

4 Dimethylamino-3-hydroxy diphenyl

4-Dimethylaminoazobenzene

Pyridium
(2 : 6 Diamino-3-phenylazopyridine)

3-(4′-Dimethylaminophenyl)azopyridine
N-oxide

Formulae C—Amines and Aminophenols

4-Dimethylaminoazobenzene or Butter Yellow is a liver carcinogen which was inactive in the bladder. 2, 6 Diamino phenyl azopyridine ochloride (Pyridium) is simi ... l other phenyl-azopyridine derivatives which Brown, Malloy, McCarthy, Verrett and Cerecedo (1951) found to be hepatic carcinogens, and is thus a heterocyclic analogue of dimethylaminobenzene. In this compound, chelation is possible through the 2-amino group and the azo linkage but dimethylaminoazobenzene which is not a bladder carcinogen could be metabolised to a hydroxy-derivative with chelating properties

2-Amino-4 . 5-dimethylphenol (2-amino-4 . 5-xylenol) is the simplest aminophenol found to be carcinogenic. Aminophenol itself and monomethyl derivatives are now being tested by the bladder implantation technique although Miller and Miller (1948) found *ortho* aminophenol to be non-carcinogenic when tested by feeding to rats.

the absorption at 298 mμ in 0·1 N-HCl solution using a Unicam Spectrophotometer. The results (Fig. 2) show that the rate of diffusion of 3-hydroxyanthranilic acid is such that about half of the 2-mg. of 3-hydroxyanthranilic acid originally present diffused out of a cholesterol pellet in 15 days and that about 10 per cent diffused out in the first 24 hours. The rate of diffusion falls with time and varies so that the diffusion at any time is proportional to the square of the amount of 3-hydroxyanthranilic acid present at that time. On the other hand only 6 per cent of the 3-hydroxyanthranilic acid was detected in the water in which paraffin wax pellets containing the acid had been incubated for 10 days.
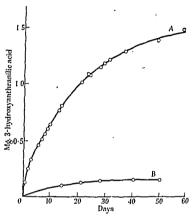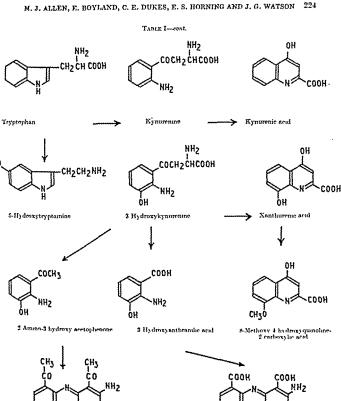
Fig. 2.—Release of 3-hydroxyanthranilic acid (3HAA) from (A) one cholesterol pellet containing 2 mg. 3HAA and (B) ten paraffin wax pellets each containing 2 mg. 3HAA.

Diffusion of the carcinogens with urine in the bladders of the mice would presumably occur at comparable rates and this could explain the apparent inactivity of the 3-hydroxyanthranilic acid in paraffin wax pellets. The diffusion of substances from paraffin wax must depend on the material first dissolving in the continuous phase of wax and then dissolving out with the surrounding water. Diffusion out must therefore depend on solubility in wax and in water.

Diffusion from cholesterol pellets can proceed either by solution in the sterol or by solution in water which penetrates between the crystals in the pellet. Polar substances which are soluble in water are therefore likely to diffuse from the compressed cholesterol pellets, but not from pellets of paraffin wax. On the other hand substances which are readily soluble in water may diffuse out of cholesterol pellets too quickly, so that they either produce toxic effects or are rapidly excreted.

TABLE I—cont.

Tryptophan   ⟶   Kynurenine   ⟶   Kynurenic acid

5-Hydroxytryptamine   3 Hydroxykynurenine   ⟶   Xanthurenic acid

3 Amino-3 hydroxy acetophenone   3 Hydroxyanthranilic acid   8-Methoxy 4 hydroxyquinoline-2 carboxylic acid

3 Amino 6 5diacetyl-phenoxazone (?)   2 Aminophenoxazone 4 5 dicarboxylic acid

Formulae II—Tryptophan Metabolites

(2) Xanthine and saccharin induced bladder tumours, but maleic hydrazide did not under these conditions.

(3) Under conditions in which 1 : 2–5 : 6-dibenzanthracene gave bladder tumours, 1 : 2–5 : 6-dibenzanthracene-3 : 4-quinone and a metabolite of the hydrocarbon, 2-phenylphenanthrene-2′ : 3-dicarboxylic acid did not induce tumours, indicating that this metabolite is not concerned in carcinogenesis.

(4) Of the sixteen metabolites which have been identified in urine of animals dosed with 2-naphthylamine, five have been tested in the mouse bladder and only one of these, 2-amino-1-naphthol glucosiduronic acid gave tumours. This is in agreement with knowledge of the behaviour of these substances.

(5) Of the simple aminophenols tested, 2-dimethylaminophenol and 2-amino-4 . 5-dimethylphenol produced tumours. 4-Dimethylaminoazobenzene, (DAB) did not induce tumours, but Pyridium (2 : 6-diamino-3-phenylazopyridine) was active.

(6) 8-Hydroxyquinoline, which like the *ortho* aminophenols is a chelating agent (which is used as spermicide and fungicide) induced cancer in the mouse bladder.

(7) Three *ortho* aminophenols which are metabolites of tryptophan—2 amino 3-hydroxyacetophenone, 3-hydroxykynurenine and 3-hydroxy anthranilic acid induced cancer on implantation in the bladders of mice. The possibility of such compounds being the cause of cancer of the bladder in man is discussed.

REFERENCES

BHARGAVA, P. M., HADLER, H. I. AND HEIDELBERGER, C.—(1955) *J. Amer. chem. Soc.*, 77, 2877.

BONSER, G. M., BRADSHAW, L., CLAYSON, D. B. AND JULL, J. W.—(1956) *Brit. J. Cancer*, 10, 531.

Idem, CLAYSON, D. AND B. JULL, J. W.—(1951) *Lancet*, ii, 286 —(1954) *Ann. Rep. Brit. Emp Cancer Compgn*, 31, 192.

Idem, CLAYSON, D. B., JULL, J. W. AND PYRAH, L. N —(1952) *Brit. J. Cancer*, 6, 412. —(1953) *Ibid* , 7, 456.

Idem AND JULL, J. W.—(1956) *J. Path. Bact.*, 72, 489.

BOYLAND, E.—(1952) *Cancer Res.*, 12, 77.—(1956) *Bull. Soc. Chim. biol* , Paris, 38, 827.

Idem AND MANSON, D.—(1955) *Biochem. J.*, 60, ii.—(1957) *Ibid* (in press). `

Idem, MANSON, D. AND ORR, S. F. D.—(1957) *Ibid.*, 65, 117.

Idem, MANSON, D., SIMS, P. AND WILLIAMS, D. C.—(1956) *Ibid.*, 62, 68.

Idem AND SIMS, P.—(1954) *J. chem. Soc.*, 980.

Idem, WALLACE, D. M. AND WILLIAMS, D. C.—(1955) *Brit. J. Cancer*, 9, 62.

Idem AND WATSON, G —(1956) *Nature*, 177, 837.

Idem AND WILLIAMS, D. C.—(1956) *Biochem. J.*, 64, 578

The inactivity of 2-amino-1-naphthol hydrochloride in the present experiments, in contrast to the activity of this substance in paraffin wax found by Bonser et al. (1956) might be due to the compound diffusing too rapidly from the cholesterol pellets.

In view of these considerations the behaviour of different substances in pellets should be studied and the appropriate medium—either paraffin wax, cholesterol or other material—chosen. The medium should allow the suspected carcinogen to be released at a suitable rate.

In these experiments there is the possibility that the action is due to some active impurity. Although attempts were made to use pure materials, many of the substances used are unstable. Thus, 3-hydroxyanthranilic acid is easily oxidised to 2-aminophenoxazone-4 : 5-dicarboxylic acid acid (Butenandt, Biekert and Neubert, 1957) and this might be a contaminant of the acid. Similarly 3-hydroxy-2-aminoacetophenone is readily oxidised to 3-amino-4 : 5-diacetyl-phenoxazone (2). These phenoxazones are similar in structure to the chromophore of actinomycin and the ammochrome pigments of insects. Derivatives of this type are under investigation for carcinogenic activity.

Plaine and Glass (1955) found that the addition of l-tryptophan indole or anthranilic acid to the diet of Drosophila melanogaster larvae produced an enormous increase in the incidence of tumours in the larvae. The effect which was even greater when the larvae were also exposed to oxygen at the same time, may be connected with the carcinogenic action of tryptophan metabolites in the bladders of mice.

Estimations of the excretion of 3-hydroxykynurenine and 3-hydroxyanthranilic acid by human subjects has shown that men with cancer of the bladder excrete more of these substances than do patients with other diseases (Boyland and Williams, 1956). These aminophenols are probably excreted in urine in con-

The induction of bladder cancer in man by this mechanism is thus thought to carcinogenic ortho aminophenols in urine from the action of enzymes. The effect can therefore concentration of the ortho aminophenol gluco-siduronide, (2) the activity of β-glucuronidase and sulphatase in the urine and (3) the time during which the urine remains in the bladder with enzymes acting on the excreted aminophenol conjugates and liberating the carcinogenic ortho aminophenols.

All these factors can be reduced unspecifically by increasing the water con-sumption and so diluting the urine. The first factor can be reduced by avoiding contact with aromatic amines or other precursors of ortho aminophenols or in some cases by correcting the diet so that phenolic metabolites of tryptophan are not excreted. The second or enzymic factor can be reduced by treatment with 1 : 4-saccharolactone which is a potent inhibitor of β-glucuronidase (Levvy, 1952).

SUMMARY

(1) The operation for implantation of pellets into the bladders of mice has been modified and used to test substances for their carcinogenic activity.

Fig. 3.—Papilloma from bladder of a mouse 40 weeks after implantation of cholesterol pellet containing 2-amino-1-naphthol glucosiduronic acid. × 25.

Fig. 4.—Section of distended bladder from a mouse 40 weeks after implantation of cholesterol pellet containing 2-amino-3-hydroxyacetophenone. × 10.

Fig. 5.—Higher magnification of focus of carcinoma from bladder shown in Fig. 4, showing small invasive focus of carcinoma. × 40.

Fig. 6.—Section of bladder of a mouse killed 40 weeks after implantation of a cholesterol pellet containing 3-hydroxyanthranilic acid showing solid differentiated carcinoma. × 11.

Fig. 7.—Section of bladder shown in Fig. 6. × 65.

Fig. 8.—Macroscopic view of the urinary bladder of a mouse killed 52 weeks following implantation of 3-hydroxyanthranilic acid in cholesterol showing papillary carcinoma. Four large carcinomatous lesions are seen projecting into the lumen of the bladder. × 3¼.

BROWN, E. V., MALLOY, P. C., McCARTHY, P., VERRET, M. J. AND CERECEDO, L. R.—(1954) *Cancer Res.*, **14**, 715.

BUTENANDT, A. AND HELLMANN, G.—(1950) *Z. Naturf.*, B56, 445.

Idem, BIEKERT, E. AND NEUBERT, G.—(1957) *Ann. Chem.*, **602**, 72.

CHARLES, R. G. AND FREISER, W.—(1952) *J. Amer. chem. Soc.*, **74**, 1385.

CLAYSON, D. B.—(1953) *Brit. J. Cancer*, **7**, 460.

DALGLEISH, C. E.—(1955) *Biochem. J.*, **61**, 334.

DARLINGTON, C. D. AND McLEISH, J.—(1951) *Nature*, **167**, 407.

FITZHUGH, O G., NELSON, A. A. AND FRAWLEY, J. P.—(1951) *J. Amer. pharm. Assoc.*, Sci. Ed., **40**, 583.

HOCH-LIGETI, C.—(1956) *Proc. Amer. Ass. Cancer Res.*, **2**, 118.

JULL, J. W.—(1951) *Brit. J. Cancer*, **5**, 328.

KIRBY, K. S.—(1956) *Biochem. J.*, **62**, 31P.

LEVVY, G. A.—(1952) *Ibid.*, **52**, 464.

MAISIN, J. AND PICARD, E.—(1924) *C.R Soc. Biol., Paris*, **91**, 799.

MILLER, J A AND MILLER, E. C.—(1948) *J. exp Med.*, **87**, 139.

Idem, MILLER, E. C., SANDIN, R. B. AND BROWN, R. K.—(1949) *Cancer Res.*, **9**, 504.

OPPENHEIMER, B. S., OPPENHEIMER, E. T., DANISHEFSKY, I., STOUT, A P AND EIRCH, F. R.—(1955) *Ibid.*, **15**, 333.
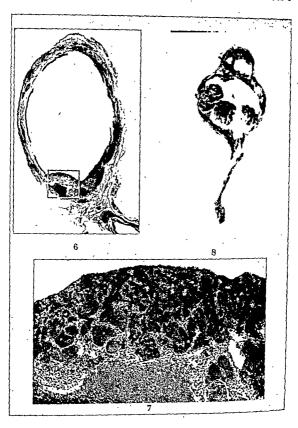
PICARD, E.—(1927) *Ann. Soc. sci. Brux.*, Ser. C., **47**, 148.

PLAINE, H. L. AND GLASS, B.—(1955) *J. Genet.* **53**, 244.
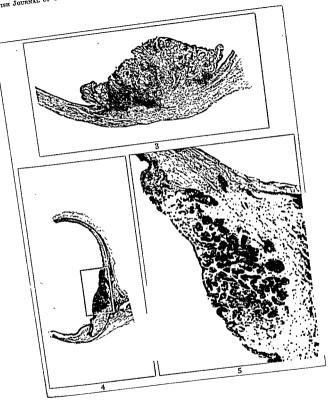
RUDALI, G., CHALVET, H. AND WINTERNITZ, F.—(1955) *C.R. Acad. Sci , Paris*, **240**, 1738.

SALAMAN, M. H. AND ROE, F. J. C.—(1956) *Brit. J Cancer*, **10**, 363.

WEGNER, E.—(1955) *Z. LebensmittUntersuch.*, **102**, 34.

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Allen, Boyland Dukes, Horning and Watson.

Allen, Boyland, Dukes. Horning and Watson

l-naphthol (2 g., m.p. 135–138° with decomposition) was added to carbon disulphide (50 ml.), dimethylaniline (10 ml.) and chlorosulphonic acid (1·4 g.). After standing overnight the solution was added to excess of 2N-KOH, extracted with ether and the aqueous phase evaporated to low volume. The *dipotassium salt of the sulphuric ester of 1-hydroxy-2-naphthylsulphamic acid* separated and was recrystallized from aqueous methanol (0·4 g., plates). (Found: N, 3·4; S, 16·5. $C_{10}H_7O_7NS_2K_2$ requires N, 3·5; S, 16·2%.) One equivalent only of chlorosulphonic acid had been employed in the hope of obtaining the unknown 1-hydroxy-2-naphthylsulphamic acid but this was not achieved. 2-Amino-1-naphthol hydrochloride gave predominantly 2-amino-1-naphthylsulphuric acid with chlorosulphonic acid (Boyland *et al.* 1953; and unpublished observations). (2) $K_2S_2O_6$ (7 g.) in water (75 ml.) was added to potassium 2-naphthylsulphamate (7 g.) and KOH (2·5 g.) in 50% aqueous acetone (250 ml.) over 1 hr. with stirring. After standing overnight, 2N-KOH (60 ml.) was added and the solution evaporated almost to dryness. The *residue was extracted several times with a total of 150 ml. of hot methanol.* After filtering and cooling the solution a mixture of product and starting material (1·5 g.) separated. Concentration to 100 ml. gave a further 1 g. of mixture and further concentration to 50 ml. gave 0·6 g. of almost pure product which after recrystallization from aqueous methanol gave the dipotassium salt of the sulphuric ester of 1-hydroxy-2-naphthylsulphamic acid. (Found: N, 3·6; S, 16·0. Calc. for $C_{10}H_7O_7NS_2K_2$: N, 3·5; S, 16·2%.) The concentration procedure and purification were guided by paper chromatography.

Products from methods (1) and (2) gave 2-amino-1-naphthol on boiling with dilute HCl, detected by the characteristic colours given when the solution was made alkaline with NH_3 soln. and extracted with benzene (Liebermann & Jacobson, 1882). The compound diazotized and coupled with hexylresorcinol gave a mauve colour but reacted only slowly with Ehrlich's reagent. It could be run without decomposition in solvent system (a) but partially decomposed in solvent system (c) to yield 2-amino-1-naphthylsulphuric acid.

[... illegible ...] The method of Smith & Williams

the compounds consisted of one molecule of amine and one molecule of *N*-glucosiduronic acid with the water of crystallization dependent on the derivative (cf. Smith & Williams, 1949b). *N*-Glucosides are also known to crystallize with water, alcohol or amine of crystallization (Mitts & Hixon, 1944).

Ammonium 2-naphthylamine N-glucosiduronate. D-[...]

(10 ml.) was added, and any precipitated amine removed by filtration and the filtrate allowed to stand for 1 hr. at room temperature, when the crystalline product (plates) was filtered off and washed successively with water, ethanol and

Table 1. R_F values and colour reactions of some aromatic amines and derivatives

All the compounds gave yellow colours with Ehrlich's reagent. For composition of solvent systems see text.

Compound	Fluorescence in ultraviolet light	Colour after diazotization and coupling with hexylresorcinol	R_F in solvent system (a)		(b)	(c)
			Descending	Ascending	Ascending	Ascending
Amine						
Phenylsulphamic acid	None	Yellow	Volatilized	—	0·75	—
2-Aminophenylsulphuric acid	None	Yellow	0·33	—	Hydrolysis	—
4-Aminodiphenyl	None	Red	0·33	—	0·66	—
4-Diphenylylsulphamic acid	Faint blue	Yellow	0·96	—	0·93	—
4-Amino-3-diphenylylsulphuric acid	Faint blue	Yellow	0·40	—	Hydrolysis	—
1-Naphthylamine	None	Red	0·47	—	0·70	—
2-Naphthylamine	Blue-white	Orange-yellow	0·54	—	0·90	—
1-Naphthylsulphamic acid	Blue	Orange-yellow	0·39	—	Hydrolysis	—
1-Amino-2-naphthylsulphuric acid	Blue-white	Blue	0·40	—	0·70	—
2-Naphthylamine	Blue-white	Yellow	0·94	0·95	0·90	0·98
2-Naphthylsulphamic acid	Blue	Yellow	0·40	0·55	Hydrolysis	0·68
2-Amino-1-naphthylsulphuric acid	Blue-white	Mauve	0·42	0·28	0·70	0·73
2-Naphthylamine-N-glucosiduronic acid	Blue-white	Yellow	—	(Partial hydrolysis)	—	Complete hydrolysis
2-Amino-1-naphthylsulphuric acid N-glucosiduronic acid	Blue-white	Mauve	0·08	0·15	—	0·30 (Partial hydrolysis)
1-Hydroxy-2-naphthylsulphamic acid sulphuric ester	Blue-white	Mauve	0·10 (Partial hydrolysis)	0·16	—	0·65 (Partial hydrolysis)

The Biochemistry of Aromatic Amines

2. THE CONVERSION OF ARYLAMINES INTO ARYLSULPHAMIC ACIDS AND ARYLAMINE-*N*-GLUCOSIDURONIC ACIDS*

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Aromatic amines are known to be metabolized by conversion into acetamido derivatives and oxidation to aminophenols. The aminophenols may be conjugated or further metabolized. In studying the metabolism of 2-naphthylamine by paper chromatography two products were detected which gave the free amine on acidification (Boyland & Manson, 1955). One of these substances could be prepared by treating 2-naphthylamine with sodium glucuronate in aqueous solution and is presumably sodium 2-naphthylamine *N*-glucosiduronate. The other product was found to be 2-naphthylsulphamic acid; this product appears to be produced in greater amounts by rabbits than by rats and an analogous reaction occurs with aniline and 1-naphthylamine. Aliphatic sulphamate or *N*-sulphonate groups are probably present in naturally occurring polysaccharides such as heparin (Reinert & Winterstein, 1939). 2-Naphthylamine and other aromatic amines may be excreted as the *N*-glucosiduronic acids and as the *N*-sulphonic acids or sulphamates.

EXPERIMENTAL

Methods

Animals were kept in metabolism cages and the urine was collected daily. Rabbits (approx. 2·5 kg.) and guinea pigs (approx. 400 g.) were fed on cabbage, bran, rat cake and water, rats (approx 200 g.) on rat cake, bread and water, and hamsters (approx. 85 g.) on rat cake, mash, mixed seeds and water. Amines were injected intraperitoneally in arachis oil. Rabbits were given 0·3 g. of aniline, 4-amino-diphenyl [...]

guinea pigs received 0·06 g. of 2-naphthylamine in 1·5 ml. of oil; hamsters received 0·02 g. of 2-naphthylamine in 0·5 ml. of oil.

Paper chromatography. Upward and downward development was employed with Whatman no. 1 chromatography paper. Solvent systems used were (*a*) *n*-butanol–propan-1-ol–0·1 N-NH₃ soln (2:1:1, by vol.) (Booth, Boyland & Manson, 1955), (*b*) benzene–methanol–amyl alcohol (B.S. 696)–2 N-HCl (35:35:17·5:12·5, by vol.) (Ekman, 1948) and (*c*) *n*-butanol–acetic acid–water (2:1:1, by vol.).

* Part 1: Boyland, Manson, Sims & Williams (1956).

Metabolites were detected on paper chromatograms by [...] with HCl then NaNO₂ (0·5%) followed by [...] colour reactions are listed in Table 1.

Materials

Melting points are uncorrected. Aniline, 4-aminodiphenyl (*p*-xenylamine), 1-naphthylamine and 2-naphthylamine were from commercial sources.

Potassium 1-amino-2-naphthylsulphate and potassium 2-amino-1-naphthyl sulphate were prepared by oxidation of 1- and 2-naphthylamine respectively with potassium [...]

Potassium 2-aminophenyl sulphate was prepared by reduction of potassium 2-nitrophenyl sulphate (Burkhardt & Wood, 1929).

Potassium salts of phenylsulphamic acid, 4-diphenylylsulphamic acid, 1-naphthylsulphamic acid and 2-naphthyl sulphamic acid were all prepared by treatment of the parent amines with chlorosulphonic acid in dry pyridine. Chlorosulphonic acid (5 g.) was added to pyridine (50 ml.) at 15°, followed by the amine (1/20 mole) and after keeping the mixture at room temperature overnight it was poured into 100 ml of N-KOH. The solution was extracted several times with ether and the aqueous phase concentrated to low volume and cooled, when the compounds crystallized out as plates. They were recrystallized from aqueous ethanol. potassium phenylsulphamate (Found: N, 6·9 Calc. for C₆H₆O₃NSK: N, 6·6%); *potassium 4-diphenylylsulphamate* (Found: N, 4·8; S, 11·4. C₁₂H₁₀O₃NSK requires N, 4·9. S, 11·15%); potassium 1-naphthylsulphamate (Found: N, 5·3. Calc. for C₁₀H₈O₃NSK. N, 5·4%); potassium 2-naphthylsulphamate (Found: N, 5·4; S, 12·5 Calc. for C₁₀H₈O₃NSK: N, 5·4, S, 12·3%).

Each gave a precipitate after slight warming with N-HCl and BaCl₂. All diazotized and coupled without prior hydrolysis but reacted only slowly with Ehrlich's reagent.

Dipotassium salt of the sulphuric ester of 1-hydroxy-2-naphthylsulphamic acid. This compound was prepared (1) by the action of chlorosulphonic acid on 2-amino-1-naphthol, and (2) by oxidation of potassium 2-naphthylsulphamate with potassium persulphate.

(1) 2-Amino-1-naphthol hydrochloride (3 g.) in water was treated with NaOH (1 equivalent) and Na₂S₂O₄ (0·5 g.) and the precipitated 2-amino-1-naphthol (plates) was washed with water and ether and dried over P₂O₅. The dry 2-amino-

(I) might be a metabolite. This was found to behave in exactly the same manner as the compound with R_F 0 6, that is, although the amino group was substituted it could be readily diazotized and coupled and reacted slowly with Ehrlich's reagent. It was readily hydrolysed on the chromatograms, even by cold mineral acid, to yield the parent amine.

(I)

2-Naphthylsulphamic acid could be detected in rabbit urine without recourse to two-dimensional chromatography although the spot overlapped with 2-amino-1-naphthylsulphuric acid. Its presence was confirmed by its isolation from rabbit urine after administration of the amine. Only small amounts were present in rat urine, in which it could be detected only by the two-dimensional technique employed above, but none was detected in the urine of guinea pigs and hamsters.

The labile precursor of 2-amino-1-naphthyl-sulphuric acid was present in the urine of rats and rabbits (the urine of hamsters and guinea pigs was not examined for this metabolite) and might be a derivative in which the amino group is substituted (II) by glucuronic acid or (III) by sulphuric acid, or

acid.

sulphuric acid could be detected so that migration of a sulphate ($-SO_3H$) group appears unlikely. If the spot due to the metabolite was cut out and extracted with methanol and the residue and the extract developed on a second chromatogram, or if the spot was cut out and run again after re-attachment to fresh paper, 2-amino-1-naphthylsulphuric acid could be detected. This decomposition on the chromatogram with the n-butanol–propan-1-ol–$0·1$ N.NH_3 soln. solvent system without any further treatment was characteristic of the synthetic N-glucosiduronic acid. Repeated running in this solvent system of a spot derived from the synthetic $O.N$-disulphate (III) did not cause any decomposition although it is hydrolysed in acetic acid–butanol systems. This metabolite is therefore 2-amino-1-naphthylsulphuric acid N-glucosiduronic acid. The corresponding spot in rabbit urine behaved in the same way.

Isolation of sodium 2-naphthylamine N-glucosiduronate. 2-Naphthylamine (0 5 g. in arachis oil) was injected intraperitoneally into each of four rabbits for 5 days (total dose 10 g.) and the urine collected and stored in the cold. The urine (2 l.) was centrifuged and the supernatant preserved for the isolation of 2-naphthylsulphamic acid (below) The deposit was washed three times with 10 ml. portions of water, flocculent material being decanted, and then washed twice with ethanol and twice with ether. The deposit was then extracted twice with hot (60°) 50% ethanol, and the extract filtered and concentrated to dryness in a desiccator at room temperature. The residue gave a rapid positive

(II)   (III)   (IV)

The precursor cannot be 2-acetamido-1-naphthyl-sulphuric acid as this has a different R_F value [0 63 in solvent system (a), upward development] and it has not been detected as a metabolite of 2-naphthylamine in rats or rabbits (unpublished observations).

The N-sulphonic acid and N-glucosiduronic acid of 2-amino-1-naphthylsulphuric acid have similar R_F values in the solvent systems employed (ascending or descending development). Both compounds yielded 2-amino-1-naphthylsulphuric acid on acid treatment of the chromatogram in the same way as the unknown metabolite, but the R_F value of the metabolite was closer to that of the N-glucosiduronic acid 1-Hydroxy-2-naphthylsulphamic acid (IV) is not known and could not be synthesized. However, after treatment of 2-hydroxyphenyl-sulphamic acid with N.HCl no 2-aminophenyl-

Tollens reaction. Paper chromatography showed the presence of a substance identical with synthetic 2-naphthylamine N-glucosiduronic acid. After three crystallizations from 50% ethanol to remove amorphous and inorganic impurities, crystalline material (1·5 mg.) was obtained, m p 180–185°.

Infrared spectral analysis (Fig. 1) also indicated that the substance was identical with synthetic sodium 2-naphthylamine N-glucosiduronate, except for some slight further absorption in the isolated sample at 1045 and 1115 cm.$^{-1}$; this must be due to the presence of an impurity in the isolated sample.

Isolation of sodium 2-naphthylsulphamate. The supernatant from the isolation of the N-glucosiduronic acid was filtered and passed through a charcoal column (approx. 150 g. of activated

ether. Yield of *ammonium 2-naphthylamine N-glucosiduro-nate*, 1·2 g., m.p 138–140° (decomp.). Recrystallization was difficult as prolonged heating with 50% ethanol caused decomposition, but a small amount was recrystallized for analysis, without altering the melting point. (Found: C, 61 9, 61·8; H, 6 45, 6 5; N, 8·4, 8·5. $C_{16}H_{20}O_6N_2,1·5H_2O$ requires C, 61·75; H, 6·4; N, 8·3%)

As the compound was insoluble in cold water, ethanol and pyridine the optical rotation could not be determined. It gave a rapid positive reaction for glucuronic acid with naphthoresorcinol solution. It was diazotized with HCl and $NaNO_2$ and the diazo compound coupled with hexyl-resorcinol. Cold 10 N-NaOH liberated NH_3. On boiling with water the material decomposed and crystals of 2-naphthyl-amine (identified by m.p. and mixed m.p.) separated on cooling. On keeping in the dry state it became brown if exposed to daylight. Paper chromatography of the substance with solvent system (a) caused some decomposition, 2-naphthylamine and the *N*-glucosiduronic acid being detected. With a two-dimensional chromatogram further decomposition occurred during the second development.

Sodium 2-naphthylamine N-glucosiduronate D-Glucurono-lactone (1·7 g.) in water (10 ml.) was converted into sodium glucuronate by addition of NaOH (0 39 g.) and allowing the solution to stand at room temperature for 3 hr. An equal volume of a phosphate buffer (pH 7 0, 0 2 M) was then added followed by 2-naphthylamine (0 7 g) in ethanol (50 ml.). The volume was made up to 100 ml with water and the solution kept at 0° overnight. Elongated plates of *sodium 2-naphthylamine N-glucosiduronate* (0 5 g) separated, the m.p. 191–192° (decomp.), being unchanged by recrystallization from aqueous ethanol.

$$[\alpha]_D^{13} -115° \xrightarrow{24\ hr.} -25°$$

(value then constant) (c, 0 08 M in water.) (Found: C, 62 0, H, 5 6; N, 6 0, 5 7, Na, 4 6, 4 8. $C_{16}H_{18}O_6N,Na,H_2O$ requires C, 62 1; H, 5 4, N, 5 6, Na, 4 6%.)

Aqueous solutions of the compound became pinkish brown in color after one …… …… ……

‡

into ammonium glucuronate as above and 2-amino-1-naphthylsulphuric acid (1 3 g., in aqueous ethanol, neutralized with dilute NH_3 soln.) was added Paper chromatography showed the presence of a substance which diazotized and coupled with hexylresorcinol to give the same mauve colour as 2-amino-1-naphthylsulphuric acid It had an R_F of 0 15 in solvent system (a) (ascending) and 0 3 in solvent system (c). The corresponding values for 2-amino-1-naphthylsulphuric acid were 0 58 …… …… …… 0 72 ……

reaction product was cut out, re-attached to fresh paper and redeveloped in solvent system (a), when both the initial

…… …… and heated at 70 for 5 min. before running again, only 2-amino-1-naphthylsulphuric acid was detectable after development of the chromatogram.

Attempts to isolate the compound were unsuccessful; gradual evaporation of the solution over P_2O_5 gave only ammonium 2-amino-1-naphthyl sulphate.

Infrared spectra

Infrared spectra of the samples as nulls in liquid paraffin or a fully fluorinated oil were measured on a Perkin-Elmer model 112 spectrometer, fitted with a NaCl prism.

RESULTS

2-Naphthylamine metabolism in rat, rabbit, guinea pig and hamster

Aromatic amines are one of a group of compounds which cause excretion of large amounts of reducing material, probably mainly glucuronic acid (Bray, Hybs & Thorpe, 1951). 2-Naphthylamine produces the same effect and the glucuronic acid excreted might react with the amine and its metabolites to yield *N*-glucosiduronic acids The reaction of *p*-toluidine with glucuronic acid has been described by Smith & Williams (1949a, b) Paper chromatography of urine from rats and rabbits injected with 2-naphthylamine showed that a spot of the same R_F and colour reactions as synthetic 2-naphthyl-amine *N*-glucosiduronic acid was present

A two-dimensional chromatogram was carried out, being developed first in solvent system (a), and the paper dried and then sprayed with N-HCl along the line of the separated metabolites The chromatogram was heated at 70° for 5 min. and dried. After treatment with ammonia vapour to neutralize residual acid (which causes distortion of the chromatogram) the sheet was developed in solvent system (c), which gives better definition of 2-naphthylamine than solvent system (a). 2-Naphthylamine was released from the spot corresponding to the *N*-glucosiduronic acid. The *N*-glucosiduronate could be detected by paper chromatography of urine immediately after it was passed. The urine was centrifuged and the deposit washed with water, ethanol and ether. This deposit gave a strong positive reaction with the Tollens test for glucuronic acid and chromatography showed the presence of the *N*-glucosiduronic acid. The presence of the compound in the deposit accords with the low solubility of the synthetic compounds. Guinea-pig and hamster urines were not examined for this compound

The two-dimensional chromatogram described above also gave 2-naphthylamine from a spot (R_F 0 6 in the first development) which diazotized and coupled with hexylresorcinol to give a yellow colour. In addition, a spot having the same colour reactions as 2-amino-1-naphthylsulphuric acid …… ……

‡

Realization that the amino group of 2-naphthyl-amine could be substituted by a very acid-labile group suggested that 2-naphthylsulphamic acid

(665)

Infrared-absorption spectra of 2-naphthylamine and the carbohydrate derivatives

The main features of the several spectra (Fig. 1) are the sharp bands between 750 and 850 cm.$^{-1}$ due to the out-of-plane deformation modes of the aromatic CH groups, the strong absorption at 950–1100 cm.$^{-1}$ in the sugar derivatives, the pair of very strong bands at about 1400 and 1600 cm.$^{-1}$ due to the carboxylic ion in the N-glucosiduronates and the strong absorption at about 1450 cm.$^{-1}$ due to the ammonium ion.

The band at about 1630 cm.$^{-1}$, present in 2-naphthylamine itself, persists in each of the sugar derivatives. Although partly due to the amino group in the amine, it must also be connected with the CC vibrations of the naphthalene nucleus, and a band at this position is present in the spectrum of 2-naphthol. If the aromatic nucleus were conjugated with a N=C bond, this band should be shifted to lower frequencies, and another band at about 1660 cm.$^{-1}$ would be expected (Bellamy, 1954a). The persistence of the band at 1630 cm.$^{-1}$ therefore suggests the presence of the –NH-C≦ structure in each of the sugar derivatives The similarity of their absorption at 745, 810 and 835 cm.$^{-1}$, due to the CH deformation modes, confirms this similarity in structure. No band due to the NH group itself could be assigned with certainty; the stretching vibration at 3 μ. is overlapped with absorption due to the sugar hydroxyl groups, and the deformation vibration does not show any well-defined characteristic absorption band (Bellamy, 1954b).

DISCUSSION

Arylsulphamic acids appear to be a new type of arylamine metabolite; these are stable in neutral solution but hydrolysed by cold acid.
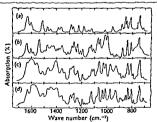
Fig. 1 Infrared spectra of (a) 2 naphthylamine, (b) 2-naphthylamine N-glucoside, (c) sodium 2-naphthylamine N-glucosiduronate and (d) ammonium 2-naphthylamine N-glucosiduronate.

Bushby & Woiwod (1955, 1956) have described the isolation of an N-glucosiduronic acid of 4 4'. diaminodiphenylsulphone from rabbit urine after administration of the parent amine. The excretion of labile glucosiduronic acids was proposed by Smith & Williams (1949a, b) to account for the presence of free glucuronic acid in the glucosiduronic acid fractions of aniline, p-phenetidine, o-, m- and p-anisidine urines. They considered the excretion of free glucuronic acid unlikely though not impossible. Bray et al. (1951) have shown, however, that reducing material, probably glucuronic acid, is present in rabbit urine after administration of several aromatic compounds. Some of this must be free and not conjugated, because the amount produced is too great to be accounted for by the dose of foreign compound administered, and the excretion continues after cessation of dosing. The formation of the N-glucosiduronic acid may occur in the urine by combination of the free amine and glucuronic acid after excretion rather than in the body tissues, but this would seem to be unlikely, particularly as Bushby & Woiwod (1956) found evidence of an N-glucosiduronic acid present in the blood of rabbits.

Booth et al. (1955) reported the formation of N-glucosides as artifacts during a study of the metabolism of 2-naphthylamine by rat-liver and kidney slices. The compounds arose by reaction of the amino and its derivatives with glucose in the Ringer solution employed.

A practical point in paper chromatographic analysis arising from the identification of N-...

This may have some importance in the identification of naturally occurring aromatic amines.

SUMMARY

1. The urine of rabbits dosed with 2-naphthylamine contains two acid-labile derivatives of 2-naphthylamine: 2-naphthylsulphamic acid and 2-naphthylamine N-glucosiduronic acid.

2. Sodium 2-naphthylsulphamate, which readily ... acid solution, has ... and detected ... of rats but not of hamsters or guinea pigs dosed with 2-naphthylamine.

3. Phenylsulphamic acid was detected in urine of rabbits but not of rats dosed with aniline. 1-Naphthylsulphamic acid was detected in the urine of rabbits but not of rats dosed with 1-naphthylamine. 4-Diphenylsulphamic acid did not appear to be formed from 4-aminodiphenyl by either rats or rabbits.

charcoal, British Drug Houses Ltd.) under slight suction. The eluate was almost colourless and showed no detectable naphthylamine metabolites. The charcoal was then washed with water (1·5 l.). This eluate also contained no detectable metabolites. Hot methanol was then passed through the column in six 500 ml. portions, which were collected separately and examined by paper chromatography. Some separation of 2-amino-1-naphthyl-sulphuric acid from 2-naphthylsulphamic acid occurred as the first and second fractions contained more of the sulphuric ester than the third and fourth, which were richer in the sulphamic acid derivative. The fifth and sixth fractions contained much less of both compounds and the sixth contained 2-acetamido-6-naphthylglucosiduronic acid (isolation of this compound will be described separately). The third, fourth and fifth fractions were combined, distilled to dryness after making alkaline with 2N-NaOH, and the gummy residue was extracted several times with hot methanol to separate inorganic material. The combined methanol extracts were evaporated to dryness, the residue was dissolved in the minimum of n-butanol–propan-1-ol–water (2:1:1, by vol.) and Whatman cellulose powder added to form a paste. The paste was added to the top of a cellulose column (23 cm. × 5 cm.) prepared in the same solvent mixture and the column developed over 18 hr., the eluate being collected in 50 ml. portions. Paper chromatography showed that the first two fractions contained 2-acetamidonaphthalene, 2-acetamido-6-naphthol and a compound believed to be 2-acetamido-5:6-dihydroxy-5·6-dihydronaphthalene. The third, fourth and fifth fractions contained 2-amino-1-naphthylsulphuric acid and 2-naphthylsulphamic acid and were evaporated to dryness and the residue was crystallized from 70% ethanol. After cooling overnight crystals (plates) of almost pure sodium 2-naphthylsulphamate were deposited which were recrystallized from 70% ethanol. Yield, 0·023 g (Found: N, 5·7; S, 13·1. Calc. for $C_{10}H_8O_3NSNa$: N, 5·7; S, 13·1%.). In another experiment 0·019 g. of sodium 2-naphthylsulphamate was isolated after injection of 6 g. of 2-naphthylamine. The R_F value was identical ... ...

... ...

hydrolysis, but it reacted slowly with Ehrlich's reagent. The compound yielded inorganic sulphate upon warming with dilute HCl. It gave a persistent yellow colour to a Bunsen flame. Hydrolysis of the compound with dilute HCl, and evaporation of the solution and treatment of the residue with benzoyl chloride in pyridine, gave 2-benzamido-naphthalene, m.p. 160–163° with no depression of m.p. with an authentic specimen of 2-benz-amidonaphthalene.

Concentration of the mother liquors of the first experiment gave only mixtures of sodium 2-amino-1-naphthyl sulphate and 2-naphthylsulphamate, which could not be separated. Cellulose-column chromatography of the material in the first and second methanol washings of the charcoal column gave mainly sodium 2-amino-1-naphthyl sulphate. To obtain the sulphuric ester all these fractions were combined and acidified with dilute HCl. The precipitate was collected and recrystallized by solution in dilute alkali and precipitation with dilute acid to yield 2-amino-1-naphthylsulphuric acid (0·049 g.), m.p. 224–226°, which gave no depression of melting point with an authentic specimen. The second experiment gave 0·029 g of the ester. 2-Amino-1-naphthylsulphuric acid has not previously been isolated from urine of rabbits injected with 2-naphthylamine.

Aniline metabolism in rabbits and rats

Phenylsulphamic acid could not be separated from 2-aminophenylsulphuric acid by paper chromatography in solvent system (a) or (c). The sulphuric ester was present in both species after administration of the amine. Aniline could not be detected after development with solvent system (a) or (c) owing to its volatility. Hence phenylsulphamic acid could not be identified (as aniline) after two-dimensional chromatography with an intermediate acid hydrolysis as described for 2-naphthylsulphamic acid. After application of the urine to the chromatogram was developed first in solvent system (a) for 18 hr. and then with solvent system (b) for 6 hr., when phenylsulphamic acid is hydrolysed but 2-aminophenylsulphuric acid is not. After treatment of rabbit urine in this manner aniline was detectable, released from a spot with an R_F value corresponding to that of phenylsulphamic acid. Phenylsulphamic acid could not be detected in rat urine.

4-Aminodiphenyl metabolism in rabbits and rats

4-Diphenylsulphamic acid and 4-amino-3-diphenylsulphuric acid were separable by paper chromatography in solvent system (a). No sulphamic acid derivative was detected in rat or rabbit urine with this solvent system or by two-dimensional chromatography in this solvent system followed by solvent system (b).

1-Naphthylamine metabolism in rabbits and rats

1-Naphthylsulphamic acid was detected in rabbit urine by the method used for the detection of 2-naphthylsulphamic acid. None was detected in rat urine.

The Biochemistry of Aromatic Amines

3. ENZYMIC HYDROXYLATION BY RAT-LIVER MICROSOMES*

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Several hydroxylating systems showing different intracellular distributions but requiring a reduced phosphopyridine nucleotide, or the nucleotide and an enzyme system capable of reducing it, have been described. Mueller & Miller (1948) reported that a rat-liver homogenate, fortified with diphosphopyridine nucleotide (DPN+), nicotinamide, magnesium ions and hexose diphosphate, demethylates and hydroxylates 4-dimethylaminoazobenzene to give 4'-hydroxy derivatives. Kensler, Miller & Hyatt (1956) showed that the hydroxylation could be carried out by the soluble liver fraction. The system responsible for the 11β-hydroxylation of 11-deoxycorticosterone by suspensions of ox-adrenocortical mitochondria requires reduced triphosphopyridine nucleotide (TPNH) (Hayano & Dorfman, 1954; Grant & Brownie, 1955); the 21-hydroxylation of 17α-hydroxyprogesterone requires the same coenzyme, but for this reaction to take place both the microsomes and the soluble fraction are needed (Ryan & Engel, 1956). Aniline and acetanilide are hydroxylated, chiefly in the p-position, by rabbit-liver microsomes and this system requires TPNH and oxygen (Brodie *et al.* 1955; Mitoma & Udenfriend, 1955; Mitoma, Posner, Reitz & Udenfriend, 1956), whereas the conversion of 2-acetamidofluorene into the 7-hydroxy derivative by rat-liver homogenate takes place in the presence of DPN+, nicotinamide and succinate (Peters & Gutman, 1956).

Two examples of naturally occurring compounds which are hydroxylated by similar systems have been reported, but in these the microsomes are not involved. A soluble enzyme system from rat liver which catalyses the conversion of phenylalanine into tyrosine requires reduced diphosphopyridine nucleotide (DPNH) and oxygen (Udenfriend & Cooper, 1952; Mitoma, 1956), and the conversion of kynurenine into 3-hydroxykynurenine by liver mitochondria of cats and rats requires TPNH (de Castro, Price & Brown, 1956).

The isolation of derivatives of 2-amino-1-naphthol and 2-amino-6-naphthol from the urine of animals dosed with 2-naphthylamine and 2-acet-

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amidonaphthalene has demonstrated that hydroxylation of the amines occurs *in vivo* (Wiley, 1938; Dobriner, Hofmann & Rhoads, 1941; Manson & Young, 1950; Boyland & Manson, 1955) and *in vitro* experiments have shown that the reaction also occurs in the presence of rat-liver slices (Booth, Boyland & Manson, 1955). The present paper reports an investigation on the enzyme system concerned with this reaction.

EXPERIMENTAL

Materials. Triphosphopyridine nucleotide (TPN+) as the dihydrate of the sodium salt, TPNH as the sodium salt, glucose 6-phosphate (G 6-P) as the sodium salt or the heptahydrate of the barium salt and glucose 6 phosphate dehydrogenase were obtained from Sigma Chemical Co. Potassium 2-naphthyl sulphamate and potassium 6-hydroxy 2 naphthyl sulphamate were prepared *by the action of chlorosulphonic acid on 2 naphthylamine and 2-amino-6-naphthol respectively [Boyland *et al.* 1957; Boyland & Manson (in preparation)] Other derivatives of 2 naphthylamine were obtained as described in a previous paper (Booth *et al* 1955) Pyrophosphate buffers were prepared from solutions of $Na_2H_2P_2O_7$ and $K_4P_2O_7$.

Liver preparations. In experiments to determine the intracellular location of the enzyme, male rat-liver homogenates in 0 25 M-sucrose were fractionated by the method of .... material was resus-

.... , chilled livers of male rats were homogenized with an MSE Ato-Mix Blender (Measuring and Scientific Equipment Co Ltd) for 0·5 min. or with a Potter & Elvehjem (1936) homogenizer in 4 vol. of 0 05 M-pyrophosphate buffer, pH 7·2. The homogenate was centrifuged for 10 min at 600 g to remove cell .... for
.... tra-
.... ium
activity in the supernatant under these conditions ....ition
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tube employed. The preparations correspond ..

4. Sodium 2-naphthylamine N-glucosiduronate was isolated from urine of rabbits dosed with 2-naphthylamine and detected in urine of rats dosed with the amine.

5. The 2-naphthylamine N-glucosiduronate was shown by infrared absorption to have a structure similar to 2-naphthylamine N-glucoside.

6. Urine of rats and rabbits dosed with 2-naphthylamine contains an acid-labile derivative of 2-amino-1-naphthylsulphuric acid. This compound is not the sulphuric ester of 1-hydroxy-2-naphthylsulphuric acid, which was synthesized, but is 2-amino-1-naphthylsulphuric acid N-glucosiduronic acid.

REFERENCES

Bellamy, L. J. (1954a). *The Infrared Spectra of Complex Molecules*, p. 227. London: Methuen.

Bellamy, L. J. (1954b). *The Infrared Spectra of Complex Molecules*, p. 219. London: Methuen.

Booth, J., Boyland, E. & Manson, D. (1955). *Biochem. J.* 60, 62.

Boyland, E. & Manson, D. (1955). *Biochem J.* 60, ii.

Boyland, E., Manson, D. & Sims, P. (1953). *J. chem. Soc* p. 3623.

Boyland, E , Manson, D., Sims, P. & Williams, D. C. (1956). *Biochem. J.* 62, 68.

Boyland, E. & Sims, P. (1954). *J. chem. Soc.* p. 980.

Bray, H. G., Hybs, Z. & Thorpe, W. V. (1951). *Biochem. J.* 48, 192.

Burkhardt, G. N. & Wood, H. (1929). *J. chem. Soc.* p. 141.

Bushby, S. R. M. & Woiwod, A J. (1955). *Amer. Rev. Tuberc.* 72, 123.

Bushby, S. R. M. & Woiwod, A. J. (1956). *Biochem. J.* 63, 406.

Ekman, B. (1948). *Acta chem. scand.* 2, 383.

Liebermann, C & Jacobson, P. (1882). *Liebigs Ann.* 211, 36.

Mitts, E. & Hixon, R. M. (1944). *J. Amer. chem. Soc* 66, 483.

Reinert, M. & Winterstein, A. (1939). *Arch. int. Pharmacodyn.* 62, 47.

Smith, J. N. & Williams, R. T. (1949a). *Biochem. J.* 44, 242.

Smith, J. N. & Williams, R. T. (1949b). *Biochem. J.* 44, 250.

Table 1. *Intracellular distribution and requirements of enzyme system responsible for the conversion of 2-acetamidonaphthalene into 2-acetamido-6-naphthol*

Flasks contain 2 mM 2-acetamidonaphthalene in a total volume of 3 ml. of pyrophosphate buffer (0 2M), pH 7 2.

Liver preparation (1 ml.)	Phosphopyridine nucleotide (0 2 mM)	Substrate for dehydrogenase (3 mM)	G 6-P dehydrogenase (0 1 %)	Gas	2 Acetamido 6-naphthol (μmoles/g of liver/hr)
Whole homogenate	TPN+	–	–	Air	1·0
Nuclei	TPN+	–	–	Air	0
Nuclei	TPNH	–	–	Air	0
Mitochondria	TPN+	–	–	Air	0
Mitochondria	TPNH	–	–	Air	0
Microsomes	TPN+	–	–	Air	0
Soluble fraction	TPN+	–	–	Air	0
Microsomes + soluble fraction	TPN+	–	–	Air	0
Microsomes + soluble fraction	TPN+	–	/	Air	1·1
Microsomes	TPNH	–	–	N_2	0
Microsomes	TPN+	G 6-P	–	Air	1 2
Microsomes	DPNH	–	–	Air	1 3
Microsome preparation	TPN+	–	~	Air	0 6
Microsome preparation	TPN+	G 6-P	–	Air	0
Microsome preparation	TPN+	G 6-P	+	Air	1 2
Microsome preparation	TPN+	Glucose	+	Air	1 2
Microsome preparation	TPN+	Malate	–	Air	1·0
Microsome preparation	TPNH	–	–	Air	0 3
Microsome preparation	DPNH	–	–	Air	0 5
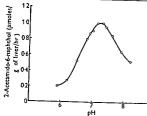
Fig. 1 Effect of pH on the rate of hydroxylation of 2-acetamidonaphthalene. Reaction mixtures (3 ml.) contained 1 ml. of microsome preparation, 3 mM-G 6-P, 0 2 mM-TPN, 35 mM-nicotinamide, 2 mM-2-acetamidonaphthalene and 0 2 M-pyrophosphate buffer, and were incubated for 30 min. at 38°.

by DPNH resulted in a loss of approximately half of the activity and complete loss of activity resulted when air was replaced by nitrogen.

Formation of 2-acetamido-6-naphthol from 2-acetamidonaphthalene

Rate of reaction and effect of pH. The rate of reaction was constant over 30 min. and was directly proportional to the volume of microsome preparation added between 0 2 and 1 ml. The estimation of activity at various pH values in the range 5·8–8·2 and with 1 ml of microsome preparation incubated for 30 min., showed optimum activity at

pH 7·2, the rate of formation of 2-acetamido-6-naphthol being 1 μmole/g. of liver/hr. (Fig 1) The activity of the microsome preparation was dependent on either TPNH or a TPNH-generating system such as TPN+, G 6-P and G 6-P dehydrogenase. Sufficient liver-G 6-P dehydrogenase was precipitated by the treatment with acetic acid, since the microsome preparation showed the same activity as a suspension of microsomes in the soluble liver fraction, and addition of G 6-P or G 6-P dehydrogenase did not increase the activity or affect the pH–activity curve.

Effect of concentrations of the reactants The rate of hydroxylation was determined in a series of experiments in which the concentration of one of the reactants was varied while the others were present in concentrations giving maximum activity. The effect of variations in TPN concentration is shown in Fig. 2, a half-maximal rate being obtained with a concentration of 0 018 mM-TPN.

The results of experiments in which the activity was measured in the presence of various concentrations of G 6-P (Fig. 3) show a half-maximal rate with 0 25 mM-G 6-P.

When the substrate concentration was varied, maximal activity occurred at a concentration of 2 mM; at higher substrate concentrations the reaction rate was diminished (Fig. 3). Examination by paper chromatography showed that some of the substrate was deacetylated to 2-naphthylamine and this compound may have inhibited the oxidation. However, estimations of 2-naphthylamine at the end of the incubations showed that the concentration was 0 07 mM, whereas a concentration of

liver/ml. and the protein content, determined by a modification of the biuret method (Fincham, 1954), was 9 mg. of protein/ml.

Estimation of hydroxylating activity with 2-acetamidonaphthalene as substrate. The activity was determined by estimating the rate of formation of 2-acetamido-6-naphthol from 2-acetamidonaphthalene. Incubations were carried out in air in flasks which were shaken for 30 min. at 38°. The standard reaction mixture consisted of 5 mM-G 6-P, 0·2 mM-TPN, 35 mM-nicotinamide, 2 mM-2-acetamidonaphthalene and 1 ml. of microsome preparation in a total volume of 3 ml. of pyrophosphate buffer (0·2M), pH 7·2. The 2-acetamidonaphthalene was added in ethylene glycol monomethyl ether (0·1 ml.), and the reaction was stopped by the addition of 10% (w/v) Na₂CO₃ (1 ml.) The reaction mixture was poured into a glass-stoppered tube, the flask washed out with n-butanol (3 ml.) and the washings were added to the tube. The addition of deacetylated _____ _____ _____ _ _

shaking and centrifuging to separate the layers, produced an orange colour in the n-butanol phase; this phase (2·5 ml.) was removed to a 5 ml. graduated tube containing 2N-NaOH (2 ml.). After shaking and allowing the layers to separate, the n-butanol layer was yellow owing to the presence of 2-naphthylamine which had been formed by deacetylation of the substrate, and the red colour produced by the 2-acetamido-6-naphthol was in the alkaline layer. This was removed (2 ml.) and added to another tube containing ethanol (0·5 ml.) to prevent turbidity, and the optical density was read at 520 mμ on a Unicam SP 500
_____ _

_____ __ _____ ___ _ ____ __

_____ ___ ____ ___ ___ _____ ___ _____ with n-butanol (3 ml.) which was then added to the supernatant and the colour developed as before.

Identification of hydroxylated metabolites of various substrates. The microsome preparation was incubated for 2 hr. in the reaction __ __ __ _

_____ substrates resulted in some of them being precipitated, and in these experiments they remained partly in suspension throughout the incubation. With 2-naphthyl-amine it was not possible ___ _ ___ _ __
wh __ _
ph __
jug _
2-amino-1-naphthylsulphuric acid could be identified.

Identification of metabolites by paper chromatography At the end of the incubation period the reaction mixture _____ _

___ approximately 18 hr. in the solvent system n-butanol-

n-propanol-water (2:1:1, by vol.). Authentic specimens of the hydroxy compounds were run alongside the metabolites in each case. The following sprays were employed for identification by colour reactions. (a) hexylresorcinol-N-HCl followed by NaNO₂ (0·5%) in 2N-NaOH); (b) 2 6-dichloroquinonechloromide (0·5% in ethanol) followed by Na₂CO₃ (10%); (c) 1-naphthol-
___ ____ __ _ _ _ _____ ___ __ ____ _

2-sulphonate in 0·1N-HCl followed by Na₂CO₃ (10%); (f) p-dimethylaminobenzaldehyde (0·5% in ethanol con-
_____ ___ __ ____ _____ __ _ _____ __

Identification of metabolites by colour tests on the reaction mixture. (a) 2-Acetamido-7-hydroxyfluorene and 2-amino-7-hydroxyfluorene were identified by the 'nitrite test' before
___ ____ _____ __ ___ _

(1 ml.), 5N-NaOH (0·5 ml.) and n-butanol (3 ml.) to the reaction mixture and shaking. The red colour produced, both by the reaction mixture and by an authentic specimen of phenol, was extracted into the n-butanol phase and had a maximum absorption at 530 mμ. The formation of phenol from benzene was confirmed with 2 6-dichloroquinone-chloromide. The reaction mixture was shaken with n-butanol (3 ml.) and centrifuged After breaking the emulsion with a glass rod and re-centrifuging, the n-butanol phase was removed. After the add ___ __ _ _f 0·5% __ 0·1 _ _

mixture and by an authentic specimen of phenol had an absorption maximum at 640 mμ. A blank in which the tissue preparation has been boiled was run at the same time in both cases. (c) p-Acetamidophenol was detected by the indophenol reaction essentially as described by Brodie & Axelrod (1948).

RESULTS

Distribution and requirements of the enzyme system

When the rat-liver homogenate was fractionated according to the method of Schneider (1948) it was found that the hydroxylating activity was present in the microsome fraction (Table I). Equal activity was obtained in the presence of either chemically prepared TPNH, TPN⁺ and the yeast liver fraction or TPN, G 6-P and G 6-P dehydrogenase. In the microsome preparations used in the quantitative experiments both the microsomes and the dehydrogenases of the liver are present, only the material soluble at pH 5 4 having been discarded. Therefore with this preparation no activity was obtained unless a substrate for one of the dehydrogenases such as G 6-P, glucose or to a less extent malate, was also added. The replacement of TPNH

The mechanism of the hydroxylation reaction is still not understood, since it is difficult to explain the requirement for TPNH in an oxidative reaction. As aromatic compounds can be hydroxylated non-enzymically in the presence of H_2O_2 and Fe^{2+} ions (Loebl, Stein & Weiss, 1949) the possibility that H_2O_2 was involved has been investigated. Then Mitoma (1956) found that H_2O_2-generating systems such as D-amino acid oxidase, uricase or xanthine oxidase and their respective substrates could not

replace the DPN$^+$ or either of the liver fractions needed for the hydroxylation of phenylalanine, similarly the TPNH required for the hydroxylation of acetanilide could not be replaced by H_2O_2 generated by glucose oxidase or D-amino acid oxidase (Mitoma et al. 1956). Furthermore, neither of these reactions was inhibited by the addition of catalase, indicating that H_2O_2 was not involved. With $^{18}O_2$ and $H_2^{18}O$ it has been demonstrated that molecular oxygen, but not water, is utilized by the

Table 2. *Compounds hydroxylated by the rat-liver microsome preparation in the presence of TPN, nicotinamide and G 6-P*

Colours were developed by spraying paper chromatograms with the reagents described in Methods unless stated otherwise. Substrates were added in ethylene glycol monomethyl ether (0·1 ml.) to give the final concentration stated, but some of the less soluble compounds were present partly in suspension.

Substrate	Hydroxylated metabolite	Method of identification	R_F	Colour
Aniline (20 mM)	o-Aminophenol	Hexylresorcinol	0·82	Mauve
		2·6·Dichloroquinonechloroimide	—	Grey-green
	p-Aminophenol	Hexylresorcinol	0·71	Red
		1-Naphthol	—	Violet
Acetanilide (2 mM)	p-Acetamidophenol	1-Naphthol after hydrolysis of reaction mixture	—	Violet
		Indophenol test on hydrolysed reaction mixture	—	Blue
2-Naphthylamine (2 mM)	2-Amino-6-naphthol	Hexylresorcinol	0·85	Red
		Diazotized sulphanilic acid	—	Mauve
		NNCD reagent (acid)	—	Mauve
		NNCD reagent (alkaline)	—	Blue
	2-Amino-1-naphthol (identified by formation of 2-amino-1-naphthyl sulphuric acid)	Hexylresorcinol	0·56	Mauve
		p-Dimethylaminobenzaldehyde	—	Yellow
2-Acetamidonaphthalene (2mM)	2-Acetamido-6-naphthol	Diazotized sulphanilic acid	0·89	Red
		2·6-Dichloroquinonechloroimide	—	Blue
2-Naphthylsulphamate (20 mM)	6-Hydroxy-2-naphthyl sulphamate	Hexylresorcinol after hydrolysis	0·21	Red
		p-Dimethylaminobenzaldehyde after hydrolysis	—	Orange
		1-Naphthol after hydrolysis	—	Puce
2-Aminofluorene (2mM)	2-Amino-7-hydroxyfluorene	p-Dimethylaminobenzaldehyde	0·76	Orange
		Nitrite test on reaction mixture	—	Yellow
2-Acetamidofluorene (2 mM)	2-Amino-7-hydroxyfluorene	p-Dimethylaminobenzaldehyde	0·76	Orange
		Nitrite test on reaction mixture	—	Yellow
	2-Acetamido-7-hydroxyfluorene	Diazotized p-nitroaniline	0·79	Blue
		Nitrite test on hydrolysed reaction mixture	—	Yellow
Benzene (20 mM)	Phenol	2·6 Dichloroquinonechloroimide test on the reaction mixture	—	Blue
		NNCD reagent test on the reaction mixture	—	Red
Naphthalene (20 mM)	1-Naphthol	Diazotized sulphanilic acid	0·97	Red
		Diazotized p-nitroaniline	—	Blue
		2·6-Dichloroquinonechloroimide	—	Blue
	1,2-Dihydronaphthalene-1,2-diol	Diazotized sulphanilic acid after hydrolysis	0·86	Red
		Diazotized p-nitroaniline after hydrolysis	—	Blue
		2·6 Dichloroquinonechloroimide after hydrolysis	—	Blue

0 25 mм-2-naphthylamıne caused only 32% in-
hibition, suggesting that it was the substrate itself
which caused a decrease in activity at concentra-
tions above 2 mм.

Hydroxylation of other aromatic compounds

The hydroxylated metabolites identified after
treating other aromatic compounds with the micro-
some preparation in the presence of TPN+, nico-
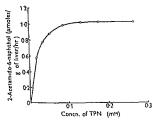
Fig 2. Effect of TPN+ concentration on the rate of hydroxyl-
ation of 2 acetamidonaphthalene   Reaction mixtures
(3 ml) contained 1 ml. of microsome preparation, 3 mм-
G 6-P, 35 mм-nicotinamide, 2 mм-2-acetamidonaph-
thalene and 0 2м-pyrophosphate buffer, pH 7 2, and were
incubated for 30 min. at 38°.
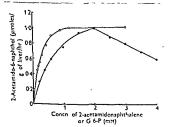
Fig. 3. Effect of G 6 P and substrate concentration on
the rate of hydroxylation of 2-acetamidonaphthalene
Reaction mixture (3 ml) contained 1 ml of microsome
preparation, 0 2 mм-TPN, 35 mм nicotinamide and 0 2м-
pyrophosphate buffer, pH 7·2, and was incubated for
30 min. at 38°. O. Hydroxylation with variations in
G 6 P concentrations in the presence of 2 mм-2-acet-
amidonaphthalene, ●. hydroxylation with variations in
2-acetamidonaphthalene concentration in the presence of
3 mм G 6 P.

tinamide and G 6-P are listed in Table 2. These
experiments were purely qualitative and the condi-
tions which produce maximum hydroxylation of
2-acetamidonaphthalene were employed.

When the three acetylated amines acetanilide,
2-acetamidonaphthalene and 2-acetamidofluorene
were used as substrates, deacetylation occurred in
each case, the free amine being identified on the
paper chromatograms. No hydroxy derivatives of
the deacetylated products, however, were identified
except 2-amino-7-hydroxyfluorene from 2-acet-
amidofluorene, but it is uncertain whether the
deacetylation or the hydroxylation occurs first. No
acetylated hydroxy compounds were found in
experiments in which the free amines were used as
substrates.

The hydroxy compounds formed from the acetyl-
ated amines acetanilide, 2-acetamidonaphthalene
and 2-acetamidofluorene were p-acetamidophenol,
2-acetamido-6-naphthol and 2-acetamido-7-hydr-
oxyfluorene respectively, the hydroxylation having
taken place at the carbon atom which is furthest
from the amino group. On the other hand, the free
amines aniline and 2-naphthylamine were oxidized
to the o-hydroxy derivatives as well. 2-Naphthyl
sulphamate appeared to behave in the same way as
2-acetamidonaphthalene in that hydroxylation
occurred in the 6-position.

The metabolites identified from naphthalene
were 1-naphthol and 1:2-dihydronaphthalene-1:2-
diol, but 2-naphthol was not seen, and phenol was
the only product identified from benzene

DISCUSSION

Since Mueller & Miller (1948) found that a rat-liver
homogenate required DPN and an oxidizable sub-
strate for the hydroxylation of 4-dimethylamino-
azobenzene in the 4'-position, the enzymic hydroxyl-
ation of several compounds has been reported, all
the reactions requiring a reduced phosphopyridine

naphthol is present in the microsomes of rat liver
and requires TPNH and oxygen. Apart from the
buffer system employed the conditions are similar
to those described by Mitoma et al. (1956) for the
hydroxylation of acetanilide by rabbit-liver micro-
somes. Although these workers reported a maxi-
mum activity at pH 8 2 for the hydroxylation of
acetanilide, whereas the pH optimum for 2-acet-
amidonaphthalene is 7·2, it would seem that the
same enzyme system is involved, and qualitative
experiments with some of the substrates used by
Mitoma et al (1956) such as aniline, acetanilide and
naphthalene yielded essentially the same meta-
bolites.

The Biochemistry of Aromatic Amines

4. O-GLUCOSIDURONIC ACID DERIVATIVES OF 2-NAPHTHYLAMINE*

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The metabolism of 2-naphthylamine is of particular interest because the amine is known to cause cancer of the bladder in man and dogs and the carcinogenic action is undoubtedly due to a metabolite excreted in urine. From the knowledge of the behaviour of the metabolites identified by earlier workers and by Boyland & Manson (1955) and Boyland, Manson & Orr (1957) it was argued (Boyland, 1956) that only one metabolite, 2-amino-1-naphthyl glucosiduronic acid, should be carcinogenic. Bladder cancer in mice has since been induced with this glucuronide (Allen, Boyland, Dukes, Horning & Watson, 1957). The preparation and properties of this and other 2-amino-naphthyl glucosiduronic acid derivatives are described in this paper.

MATERIALS

2-Naphthylamine, 2-acetamido-1-naphthol, 2-acetamido-6-naphthol hydrochloride were prepared as described by Booth, Boyland & Manson (1955). Calf-spleen β-glucuronidase (Viobin Laboratories, Monticello, Ill., U.S.A.) and bacterial β-glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) were employed for enzymic hydrolyses

METHODS

Rats and rabbits were kept in metabolism cages and urine was collected daily. Rabbits were fed on cabbage, bran, rat cake and water, and rats on bread, rat cake and water. 2-Naphthylamine or 2-acetamidonaphthalene was injected intraperitoneally in daily doses of 0·5 g. in 10 ml. of arachis oil into rabbits and 0·05 g. in 1 ml. of oil into rats Paper chromatography was carried out by upward development on Whatman no 1 paper with butanol–propanol–aqueous 0·1N-NH$_3$ (2.1 1, by vol). Acetamido compounds were hydrolysed to aminocompounds on the chromatograms by spraying with N HCl and heating for 30 min. between glass plates at 70°. The colour reactions of acetamidonaphthol glucosiduronic acids after hydrolysis under such conditions are characteristic of the aminonaphthyl glucosiduronic acids and not of the aminonaphthols. Reagents used in this work have been described by Booth et al. (1955). 2-Amino-1-naphthol was detected in solution by the green-coloured derivative which is formed on addition of NH$_3$. This derivative is extracted with benzene, in which solvent

it is mauve (Liebermann & Jacobson, 1882). A Chromatolite lamp (Hanovia Ltd.) was used as a source of ultraviolet light R_F values and colour reactions of the compounds isolated are given in Table 1. R_F values obtained by downward development in several solvent systems and colour reactions with nitrous acid followed by hexyl-resorcinol and with Ehrlich's reagent have been given by Booth et al. (1955).

RESULTS

ISOLATION OF GLUCOSIDURONIC ACIDS

Both 2-amino-1-naphthol hydrochloride and the free base are toxic (LD$_{50}$ in mice 25 mg./kg body wt.) and so unsuitable as sources of 2-amino-1-[...] [...] [...] [...] acid The administration [...] [...] [...] [...] h [...] [...])-1-naphthol was therefore used and the acetamido group of the glucosiduronic acid was hydrolysed by dilute sulphuric acid. Attempts to isolate 2-amino-1-naphthyl glucosiduronic acid from the urine of rats dosed with 2-naphthylamine were unsuccessful, although the acid could be detected in the urine by paper chromatography. Small amounts of 2-amino-6-naphthyl glucosiduronic acid were isolated from the urine of rabbits dosed with 2-amino-6-naphthol.

2-Acetamido-6-naphthyl glucosiduronic acid was isolated as its sodium salt from rabbit urine after administration of 2-acetamidonaphthalene. Rabbits deacetylate acetyl derivatives of arylamines relatively slowly, and when dosed with 2-acetamidonaphthalene rabbits excrete only small amounts of 2-amino-1-naphthylsulphuric acid and neither 2-naphthylsulphamic acid nor 2-amino-1-naphthyl glucosiduronic acid. 2-Acetamido-6-naphthyl glucosiduronic acid was, however, isolated from rabbit urine as the triacetyl methyl ester after injection of 2-naphthylamine. The glucosiduronic acid fraction was obtained by adsorption on charcoal and elution. If the charcoal was heated before use (cf. Corner, Billett & Young, 1954) from rabbit urine acid was bound so that only small amounts were removed with cold or hot methanol, but it was eluted with aqueous phenol (cf. Dalgliesh, 1952) If the

adrenal gland in the 11-β-hydroxylation of steroids (Hayano, Lindberg, Dorfman, Hancock & Doering, 1955). Further evidence for the utilization of molecular oxygen was provided by the finding that the oxygenation of the hydroxyl group which is introduced into salicylate by the model hydroxylase ferrous ion, ethylene diaminetetra-acetate, oxygen and ascorbate (Brodie, Axelrod, Shore & Udenfriend, 1954) arose from molecular oxygen (Mason & Onoprienko, 1956), and Mason, Fowlks & Paterson (1955) have shown that molecular oxygen contributes directly in phenolase-catalysed hydroxylations of some aromatic compounds. It has in fact been suggested (Velick, 1956) that phenolase may provide a model for the TPNH and oxygen-requiring systems, since if molecular oxygen and oxidizable metallo-enzymes were involved the TPNH would serve to regenerate the reduced and active enzyme. Both the hydroxylation of phenylalanine and acetanilide are inhibited by the ferrous ion-complexing agent, αα'-dipyridyl (Mitoma, 1956; Mitoma et al. 1956), whereas the 11β-hydroxylation of 11-deoxycorticosterone is strongly inhibited by diethyl dithiocarbamate (Sweat et al. 1956), suggesting that the enzymic activity in this case is associated with a copper complex.

Although naphthalene is converted into both 1-naphthol and 1:2-dihydronaphthalene-1·2-diol by this system, it seems likely that two different enzyme systems having the same location and requiring the same coenzyme are responsible for these two different types of reactions. 1:2-Dihydronaphthalene1:2-diol is readily dehydrated to 1-naphthol by mineral acid, but when it is incubated with the enzyme system no 1-naphthol can be detected, so that 1·2-dihydronaphthalene-1.2-diol is unlikely to be an intermediate between naphthalene and 1-naphthol. A further study of these two reactions may throw some light on the hydroxylation mechanism.

SUMMARY

1. The enzyme system responsible for the conversion of 2-acetamidonaphthalene into 2-acetamido-6-naphthol is associated with the microsomes of rat liver and requires reduced triphosphopyridine nucleotide (TPNH) and oxygen

2. The TPNH can be replaced by triphosphopyridine nucleotide, glucose 6-phosphate (G 6-P) and G 6-P dehydrogenase, and conditions affecting the rate of hydroxylation of 2-acetamidonaphthalene by this system have been studied

3. The system is not specific for 2-acetamidonaphthalene but will hydroxylate other aromatic amines, N-substituted amines and also benzene and naphthalene

REFERENCES

Bernstein, S. & McGilvery, R. W. (1952). J. biol. Chem. 198, 195.

Booth, J., Boyland, E. & Manson, D. (1955) Biochem. J. 60, 62.

Boyland, E. & Manson, D. (1955). Biochem. J. 60, ii.

Boyland, E., Manson, D. & Orr, S. F. D. (1957). Biochem. J. 65, 417.

Brodie, B. B. & Axelrod, J. (1948). J. Pharmacol. 94, 22.

Brodie, B. B, Axelrod, J. Cooper, J. R., Gaudette, L., La Du, B. N., Mitoma, C. & Udenfriend, S. (1955). Science, 121, 603

Brodie, B. B, Axelrod, J, Shore, P. A. & Udenfriend, S. (1954). J. biol. Chem. 208, 741.

Damron, C. M & Dyer, H. M. (1953). J. nat. Cancer Inst. 14, 279.

de Castro, F. T., Price, J. M. & Brown, R. R. (1956) J. Amer. chem. Soc. 78, 2904.

Dobriner, K., Hofmann, K. & Rhoads, C. P. (1941) Science, 93, 600.

Fincham, J. R. S. (1954). J. gen. Microbiol. 11, 236.

Grant, J. K. & Brownie, A. C. (1955). Biochim biophys. Acta, 18, 433.

Hayano, M. & Dorfman, R. I. (1954). J biol, Chem. 211, 227.

Hayano, M., Lindberg, M. C., Dorfman, R. I., Hancock, J. E. H. & Doering, W. von E. (1955). Arch Biochem. Biophys. 59, 529.

Kensler, C. J, Miller, A. & Hyatt, M. (1956). Proc. Amer. Ass. Cancer Res 2, 124.

Loebl, H., Stein, G. & Weiss, J (1949). J. chem. Soc. p 2074.

Manson, L. A & Young, L. (1950). Biochem. J. 47, 170.

Mason, H. S., Fowlks, W L & Paterson, E. J. (1955). J Amer. chem. Soc. 77, 2914.

Mason, H S & Onoprienko, I. (1956). Fed. Proc. 15, 310

Mitoma, C. (1956). Arch. Biochem. Biophys. 60, 476.

Mitoma, C., Posner, H S, Reitz, H. C. & Udenfriend, S. (1956). Arch Biochem Biophys 61, 431

Mitoma, C. & Udenfriend, S. (1955). J Pharmacol. 113, 40.

Mueller, G C. & Miller, J C (1948). J biol Chem 176, 535

Peters, J H & Gutman, H R. (1956) Arch Biochem. Biophys. 62, 234

Potter, V. R & Elvehjem, C A (1936) J biol Chem 114, 4951.

Ryan, K. J. & Engel, L. L (1956). J. Amer chem Soc. 78, 2654

Schneider, W C (1948) J. biol Chem 176, 259

Sweat, M L., Aldrich, R A, de Brum, C. H., Fowlks, W. L, Heiselt, L R. & Mason, H. S. (1956) Fed. Proc. 15, 367.

Udenfriend, S. & Cooper, J R. (1952) J. biol Chem 194, 503

Velick, S F. (1956). Annu Rev. Biochem. 25, 277

Wiley, F. H. (1938) J. biol Chem 124, 627

5.6-dihydroxynaphthalene and a trace of 2-acetamido-6-naphthyl glucosiduronic acid Elution with 5% aqueous phenol removed the 2-acetamido-6-naphthyl glucosiduronic acid.

The charcoal was stirred with hot methanol (total vol. 3 l.) until all the first series of metabolites were extracted. The charcoal was then packed in a column above a layer of sand and treated with 5% aqueous phenol until the glucosiduronic acids were eluted, as indicated by paper chromatography. The eluate was evaporated to dryness and then kept in an evacuated desiccator over sodium hydroxide pellets for 24 hr. to remove the residual phenol. Solution of the residue in the minimum of water and the addition of several volumes of ethanol gave a hygroscopic precipitate (4 g.). {A sample of this preparation was incubated with bacterial β-glucuronidase in acetate buffer at pH 6 0 for 24 hr. at 37°, paper chromatography showed that 2-acetamido-6-naphthol and a little of the presumed 2-acetamido-5.6-dihydro-5:6-dihydroxynaphthalene had been liberated.} After three crystallizations by solution in the minimum of hot water and addition of hot ethanol sodium 2-acetamido-6-naphthyl glucosiduronate (1·0 g.) was obtained as needles, m.p. 252-254° (decomp.), $[\alpha]_D^{25} - 78.4°$ in water (c, 1·02) {Found: C, 49·5; H, 5·05; N, 3·2; Na, 5 8. $C_{18}H_{18}O_8NNa,2H_2O$ requires C, 49 65; H, 5·1; N, 3 2; Na, 5·3%}. The yield was equivalent to 2·5% of the dose. The compound dissolved readily in water, giving a neutral solution, and was soluble in methanol and insoluble in ethanol. It gave a positive reaction with naphthoresorcinol and diazotized and coupled only after heating with $2N$-HCl. After incubation with bacterial β-glucuronidase at pH 6 0 (acetate buffer) or pH 7 0 (phosphate buffer) 2-acetamido-6-naphthol was detected by paper chromatography but 2-acetamido-5 6-dihydro-5 6-dihydroxynaphthalene was not present. The hydrolysis product was isolated, m p. alone and mixed with an authentic specimen of 2-acetamido-6-naphthol, 221–223°.

Sodium 2-acetamido-6-naphthyl glucosiduronate (0 4 g.) on heating for 3 hr at 100° in $5N$-H_2SO_4 (10 ml.) gave 2-amino-6-naphthol, confirmed by the red colour given on diazotization and coupling with hexylresorcinol. The hydrolysis product was acetylated with acetic anhydride in pyridine and the diacetyl derivative, after recrystallization from ethanol, melted at 217–220°, alone and mixed with an authentic specimen of 2-acetamido-6-acetoxynaphthalene. Acidification of an aqueous solution of sodium 2-acetamido-6-naphthyl glucosiduronate gave no crystalline material and evaporation of the solution gave a yellow gum, soluble in water and ethanol, which could not be crystallized.

p-Toluidine 2-acetamido 6-naphthyl glucosiduronate was prepared by the addition of *p*-toluidine hydrochloride (0·1 g) to a solution of the sodium salt (0·1 g.) in hot water (5 ml.). On cooling, needles separated which after recrystallization from water melted at 181–182° (decomp), $[\alpha]_D^{25} - 72.5°$ in water (c, 1 02) {Found: C, 62 0; H, 5 7; N, 5·8 $C_{25}H_{26}O_8N_2$ requires C, 62 0, H, 5 8; N, 5 8%}

In another experiment charcoal which was not preheated was used for adsorption and exhaustively extracted with   with 2-acet   re portions,   ed through   eluted with   Indoxyl-sulphuric acid was identified in the methanol fractions. The

columns were lagged and eluted with hot methanol (about 60°) in 500 ml. portions. Urinary phenols were eluted in the first three fractions. Twelve hot methanol washings were collected; the later washings contained very little 2-acetamido-6-naphthyl glucosiduronic acid.The hot methanol washings were combined, evaporated almost to dryness and the residue was dissolved in water and extracted several times with ether, followed by ethyl acetate. Ethyl acetate removes the compound which is thought to be 2-acetamido 5:6-dihydro-5 6-dihydroxynaphthalene. The aqueous fraction was again evaporated to dryness and the residue dissolved in the minimum of butanol-propanol-water (2:1:1, by vol) and sufficient Whatman cellulose powder added to form a paste. The paste was added to the top of a cellulose column (30 cm. × 6 cm.) prepared with the same solvent mixture and was slowly eluted over 5 days Collection of fractions was guided by paper chromatography. 2-Acetamido-6-naphthol, 2-acetamido 5 6-dihydro 5 6-dihydroxynaphthalene, 2-acetamido-6-naphthylsulphonic acid, an unidentified sulphuric ester and the glucosiduronic acid fraction were successively eluted from the column. Enzyme hydrolyses showed that this last fraction contained the glucosiduronic acids of 2-acetamido-6-naphthol and 2-acetamido-5 6-dihydro 5 6-dihydroxynaphthalene, but mainly the former compound. The glucosiduronic acid fractions were combined and evaporated to dryness, the residue dissolved in the minimum of hot water and hot ethanol added until the solution was just cloudy. On cooling, rosettes of needles (3 5 g) were formed, which after recrystallization gave sodium 2-acetamido-6-naphthyl glucosiduronate (1·5 g.) m p 252-254°. A further 1 0 g of less pure material was obtained from the mother liquors and another 1·0 g. by elution of the charcoal with aqueous phenol. Attempts to isolate 2-acetamido-5 6-dihydro 5 6 dihydroxynaphthalene glucosiduronic acid by chromatography of the mother liquors or by fractional crystallization of the triacetyl methyl esters were not successful.

(b) As the p-toluidine salt. Urine from rabbits dosed with 2-acetamidonaphthalene (14 g.) was passed through a column of charcoal. The column was washed with water, with cold methanol which eluted relatively little of the glucuronide, and finally with aqueous phenol. The phenolic   in the
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coal column (150 g , not pretreated by heat). The column
                     ith hot methanol until
                     and then
                     at from
                     usted to
                     iccator.
                     treated
with an ethereal solution of diazomethane (from 20 g. of nitrosomethylurea) and kept overnight at 5°. After filtration and concentration in vacuo the residue was dissolved in pyridine (10 ml) and acetic anhydride (10 ml.). The solution was kept overnight and poured into water, and the precipitate

Table 1. R_F values and colour reactions of 2-amino- and 2-acetamido-naphthyl glucosiduronic acids

Conditions of hydrolysis are described in the text.

Compound	R_F in butanol-propanol-0·1 N-NH$_3$ (2:1·1, by vol)	Fluorescence	Colours with diazotized sulphanilic acid	
			Before hydrolysis	After hydrolysis
	0·22	Blue–white	Pale yellow	Pale yellow
	0·25	None	None	Pale yellow
	0·15	Blue–white	Orange-red	Orange-red
	0·20	None	None	Orange-red

charcoal was not heated, cold methanol eluted only a little of the glucosiduronic acid; hot methanol removed more, but aqueous phenol was still necessary for complete elution. The glucosiduronic acid fractions contained 2-acetamido-6-naphthyl glucosiduronic acid, with small amounts of what is probably 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid. Evidence for the presence of the latter conjugate is discussed below.

2-Acetamido-1-naphthyl glucosiduronic acid

2-Acetamido-1-naphthyl glucosiduronic acid (0·3 g. in 10 ml of arachis oil) was administered daily for 3 days to each of five rabbits (total dose 4·5 g.). The urine was adjusted to pH 4·0–5·0 with acetic acid and a saturated solution of lead acetate added. After removal of the precipitate by centrifuging, the supernatant was adjusted to pH 8·0 with aq. NH$_3$ (sp.gr. 0·88) and a saturated solution of basic lead acetate added until precipitation was complete. The precipitate was

... talao. On cooling, plates of *2-acetamido-1-naphthyl glucosiduronic acid* were deposited, which after crystallization from water had m.p. 194–197°, $[\alpha]_D^{25} + 2·4°$ in ethanol (c, 3·7) (Found: neutralization equivalent, 408; C, 52·4, 52·2, H, 5·8, 5·8; N, 3·4, 3·6%. C$_{18}$H$_{19}$O$_8$N,2H$_2$O requires neutralization equivalent 413; C, 52·2; H, 5·6, N, 3·4%). On heating with 5 N-HCl for 15 min. a compound was formed which after diazotization could be coupled with hexyl-resorcinol...

of 1·5 g. of the compound was isolated, equivalent to 16% of the dose. The urine collected for a further 2 days yielded only a few milligrams of the glucosiduronic acid.

Examination of the untreated urine by paper chromatography showed that free 2-acetamido-1-naphthol and traces of 2-amino-1-naphthylsulphuric acid and 2-amino-1-naphthyl glucosiduronic acid were present, but no 2-acetamido-1-naphthylsulphuric acid could be detected.

2-Amino-1-naphthyl glucosiduronic acid

2-Acetamido-1-naphthyl glucosiduronic acid (1·0 g.) was heated on a water bath with 0·5 N-H$_2$SO$_4$ (15 ml.) for 30 min., and the solution was treated with a small amount of charcoal, filtered and kept at room temperature overnight. Unchanged acetamido derivative (0·5 g.) separated and was filtered off; the filtrate was adjusted to pH 4·0 and evaporated in a vacuum desiccator until brown crystals began to

appear. The solution was cooled to 5° overnight and the product filtered off. Recrystallization from water yielded small rosettes of prisms (100 mg., 11%) of *2-amino-1-naphthyl glucosiduronic acid*, m.p. 178–180° (decomp.), $[\alpha]_D^{15} - 155°$ in 0·1 N-HCl (c, 0·175) (Found: C, 54·4, H, 5·8; N, 4·3, 4·2. C$_{16}$H$_{17}$O$_7$N,H$_2$O requires C, 54·4; H, 5·4; N, 4·0%). The compound was insoluble in water and readily soluble in ethanol. After hydrolysis by calf-spleen β-glucuronidase, 2-amino-1-naphthol was detected by the colour produced on shaking with aq. NH$_3$.

The acid hydrolysis gave some 2-acetamido-1-naphthol and for this reason the hydrolysis time was limited to 30 min. A guide to the optimum time of hydrolysis was obtained by examining samples by paper chromatography at intervals. Hydrolysis of the glucosiduronic acid linkage was slower than that of the acetamido group. Attempted hydrolysis with 2 N-H$_2$SO$_4$ or 0·2 N-HCl caused blackening and 2 N-NaOH gave a red gum from which no 2-amino-1-naphthyl glucosiduronic acid could be isolated.

2-Amino-6-naphthyl glucosiduronic acid

Each of five rabbits received 2-amino-6-naphthol hydrochloride (0·45 g.), neutralized just before injection, for 4 days (total dose 7·2 g. of 2-amino-6-naphthol). The glucosiduronic acid fraction was separated by the lead acetate method and dissolved in the minimum of water, and the solution adjusted to pH 5·0. On cooling to 5° crystals of *2-amino-6-naphthyl glucosiduronic acid* separated after 2 days. Recrystallization by solution in 2 N-HCl and adjustment of the pH to 5·0 yielded 0·06 g. (0·4%) of theory), $[\alpha]_D^{19} - 76·8°$ in 0·1 N-HCl (c, 0·625). The compound blackened and sintered at 250° (Found: C, 54·2; H, 5·55; N, 4·0. C$_{16}$H$_{17}$O$_7$N,H$_2$O requires C, 54·4; H, 5·4, N, 4·0%). Paper chromatography showed that the mother liquors contained the acetamido derivative in addition to the amino compound.

2-Acetamido-6-naphthyl glucosiduronic acid

(a) *As the sodium salt.* 2-Acetamidonaphthalene was given daily to each of six rabbits for 7 days (total dose 16·8 g.). The collected urine was filtered (Ford's clarifying pad, grade F.C.B.) and adjusted to pH 7·0. Each day's urine was stirred for 0·5 hr. with about 50 g. of charcoal (British Drug Houses Ltd. activated charcoal, heated at 150° for about 0·5 hr. and cooled) and filtered. The almost colourless filtrate contained no detectable metabolites of 2-acetamidonaphthalene, but still gave a strong naphthoresorcinol reaction.

Preliminary experiments showed that elution of the charcoal with water or aq. 0·1 N-NH$_3$ did not remove metabolites but that elution with hot methanol removed 2-acetamido-6-naphthol. 2-acetamido-6-naphthylsulphuric acid, a compound considered to be 2-acetamido-5:6-dihydro-

5,6-dihydroxynaphthalene and a trace of 2-acetamido-6-naphthyl glucosiduronic acid. Elution with 5% aqueous phenol removed the 2-acetamido-6 naphthyl glucosiduronic acid.

The charcoal was stirred with hot methanol (total vol. 3 l.) until all the first series of metabolites were extracted. The charcoal was then packed in a column above a layer of sand and treated with 5% aqueous phenol until the glucosiduronic acids were eluted, as indicated by paper chromatography. The eluate was evaporated to dryness and then kept in an evacuated desiccator over sodium hydroxide pellets for 24 hr. to remove the residual phenol. Solution of the residue in the minimum of water and the addition of several volumes of ethanol gave a hygroscopic precipitate (4 g.). (A sample of this preparation was incubated with bacterial β-glucuronidase in acetate buffer at pH 6 0 for 24 hr. at 37°; paper chromatography showed that 2-acetamido-6-naphthol and a little of the presumed 2-acetamido-5:6-dihydro-5,6 dihydroxynaphthalene had been liberated.) After three crystallizations by solution in the minimum of hot water and addition of hot ethanol sodium 2-acetamido-6 naphthyl glucosiduronate (1 0 g.) was obtained as needles, m.p. 252–254° (decomp.), $[\alpha]_D^{20}$ −78 4° in water (c, 1 02) (Found: C, 49 5; H, 5 05; N, 3 2; Na, 5·8. $C_{18}H_{18}O_8NNa.2H_2O$ requires C, 49 65; H, 5·1; N, 3 2; Na, 5 3%). The yield was equivalent to 2·5% of the dose. The compound dissolved readily in water, giving a neutral solution, and was soluble in methanol and insoluble in ethanol. It gave a positive reaction with naphthoresorcinol and diazotized and coupled only after heating with 2 N·HCl. After incubation with bacterial β-glucuronidase at pH 6 0 (acetate buffer) or pH 7·0 (phosphate buffer) 2-acetamido 6-naphthol was detected by paper chromatography but 2-acetamido-5 6-dihydro-5,6-dihydroxynaphthalene was not present. The hydrolysis product was isolated, m.p. alone and mixed with an authentic specimen of 2-acetamido-6-naphthol, 221–223°.

diazotization and coupling with hexylresorcinol. The hydrolysis product was acetylated with acetic anhydride in pyridine and the diacetyl derivative, after recrystallization from ethanol, melted at 217–220°, alone and mixed with an authentic specimen of 2-acetamido-6-acetoxynaphthalene. Acidification of an aqueous solution of sodium 2-acetamido-6-naphthyl glucosiduronate gave no crystalline material and evaporation of the solution gave a yellow gum, soluble in water and ethanol, which could not be crystallized.

p-Toluidine 2-acetamido-6-naphthyl glucosiduronate was prepared by the addition of p toluidine hydrochloride . . . .

$[\alpha]_D^{24}$ −72 5° in water (c, 1·02) (Found: C, 62 0; H, 5 7; N, ...

amidonaphthalene (28 g.) was divided into five portions, each approximately 2 l., which were each passed through columns of charcoal (250 g.). The columns were eluted with water (2 l.) and cold methanol (3 × 500 ml). Indoxyl-sulphuric acid was identified in the methanol fractions. The

columns were lagged and eluted with hot methanol (about 60°) in 500 ml. portions. Urinary phenols were eluted in the first three fractions. Twelve hot methanol washings were collected; the later washings contained very little 2-acetamido-6-naphthyl glucosiduronic acid The hot methanol washings were combined, evaporated almost to dryness and the residue was dissolved in water and extracted several times with ether, followed by ethyl acetate. Ethyl acetate removes the compound which is thought to be 2-acetamido-5:6-dihydro-5,6-dihydroxynaphthalene. The aqueous fraction was again evaporated to dryness and the residue dissolved in the minimum of butanol–propanol–water (2:1:1, by vol) and sufficient Whatman cellulose powder added to form a paste. The paste was added to the top of a cellulose column (30 cm. × 6 cm.) prepared with the same solvent mixture and was slowly eluted over 5 days. Collection of fractions was guided by paper chromatography. 2-Acetamido 6-naphthol, 2-acetamido-6-naphthol, 2-acetamido-5 6-dihydroxy-naphthalene, 2 acetamido 6-naphthylsulphuric acid, an unidentified sulphuric ester and the glucosiduronic acid fraction were successively eluted from the column. Enzymic hydrolyses showed that this last fraction contained the glucosiduronic acids of 2 acetamido-6-naphthol and 2-acetamido-5 6-dihydro-5 6-dihydroxynaphthalene, but mainly the former compound. The glucosiduronic acid fractions were combined and evaporated to dryness, the residue dissolved in the minimum of hot water and hot ethanol added until the solution was just cloudy. On cooling, rosettes of needles (3 5 g) were formed, which after recrystallization gave sodium 2-acetamido-6-naphthyl glucosiduronate (1·5 g.) m.p. 252–254°. A further 1·0 g of less pure material was obtained from the mother liquors and another 1 0 g. by elution of the charcoal with aqueous . . . . glucuronide, and finally with aqueous phenol. The pneumo . . . . did not depress the m p of the derivative prepared . . . . .

collected. After two crystallizations from aqueous ethanol
[faded line] ....

$C_{20}H_{27}O_{11}N$ requires C, 58·0, H, 5·3; N, 2·7%). Although the
original crude solid appeared to contain 2-acetamido-5·6-
dihydro-5·6-dihydroxynaphthalene glucosiduronic acid no
crystalline triacetyl methyl ester of this could be isolated.

Isolation of allantoin

The first and second cold methanol eluates from charcoal
columns (see p. 277) were combined and the solution was
concentrated to 30 ml. On cooling, prisms (1·5 g. from 10 l.
of urine) separated, m.p. after three crystallizations from
water 234–236° (decomp.), alone and mixed with an
authentic specimen of allantoin (Found. N, 35·6. Calc. for
$C_4H_6O_3N_4$: N, 35·4%) The compound had an R_F of 0·1
in butanol–propanol–0·1 N-NH$_3$, identical with that of
allantoin. The spot gave a yellow colour with Ehrlich's
reagent and a green colour with sodium hypochlorite (5% in
water) after spraying with phenol (5% in 95% ethanol) On
heating either the isolated substance or the authentic
allantoin in 5 N-HCl with naphthoresorcinol it gave a
cherry-red colour, extractable by ether. Asher (1910)
described this colour reaction of allantoin and the adsorption
of allantoin by charcoal. Corner & Young (1955) described
the formation of a reddish purple colour from an unidentified
constituent with naphthoresorcinol during the estimation of
glucuronic acid in rat urine, but the colour did not interfere
with the estimations. It is possible that allantoin was a
contribut... f... . .. . f..... ... .f ... .

The O-glucosiduronic acids formed in the metabolism of 2-naphthylamine

After rats and rabbits had been dosed with 2-
naphthylamine the urine of each species was
examined by paper chromatography for the presence
of glucosiduronic acids. 2-Amino-1-naphthyl gluco-
siduronic acid was detected in the urine of both
species, but was more abundant in that of the rat.
Rat urine (2 ml.), diluted with an equal volume of
0·2 M-acetate buffer (pH 4·5), was incubated with
calf-spleen β-glucuronidase at 37° for 2 days
2-Amino-1-naphthol was liberated and identified by
the ammonia–benzene test. A control urine sample
gave no colour reaction to this test. A fraction
containing the compound could be obtained by
precipitation of the urine of rats or rabbits by basic
lead acetate.

2-Amino-6-naphthyl glucosiduronic acid was
detected in rat urine but not in rabbit urine. The
compound was precipitated from rat urine by basic
lead acetate After decomposition of the lead salt
by hydrogen sulphide a sample of the glucosiduronic
acid fraction so obtained gave 2-amino-6-naphthol
on incubation with calf-spleen β-glucuronidase.
Rabbit urine, but not rat urine, contained a com-
pound (R_F 0·2) which was non-fluorescent and
which was not diazotizable until after treatment

with acid at 70°. It corresponded in its reactions to
2-acetamido-6-naphthyl glucosiduronic acid. Paper
chromatography of the urine after it had been
heated for 15 min. with an equal volume of 5 N-
hydrochloric acid gave a new spot which corre-
sponded in R_F and colour reactions to 2-amino-6-
naphthyl glucosiduronic acid. Although only one
glucosiduronic acid appeared on hydrolysis there
are two possibilities for the structure of its pre-
cursor. Both 2-acetamido-6-naphthyl glucosid-
uronic acid and 2-acetamido-5·6-dihydro-5:6-di-
hydroxynaphthalene glucosiduronic acid would
give the amino compound on acid treatment. The
glucosiduronic acid fraction of rabbit urine (50 ml.)
was prepared by precipitation with basic lead
acetate. After decomposition of the lead salt by
hydrogen sulphide excess of the latter was removed
by a current of air. The solution was adjusted to
pH 6·5 and continuously extracted with ether for
18 hr and then evaporated to 5 ml. in a desiccator
at room temperature. After dilution with an equal
volume of acetate buffer (pH 6·0) a sample of the
solution was incubated with bacterial β-gluc-
uronidase. Paper chromatography showed that two
new spots were present. One corresponded to
2-acetamido-6-naphthol in R_F and colour
reactions. The other spot (R_F 0·81) was not fluor-
escent, did not react with diazotized sulphanilic
acid, could not be diazotized and coupled with
hexylresorcinol, but after treatment with acid at
70° the material in the spot could be diazotized
(green colour with nitrous acid) and coupled with
hexylresorcinol (red colour), and coupled with
diazotized sulphanilic acid (mauve colour)—re-
actions identical with those of 2-amino-6-naphthol.

The material obtained by enzymic hydrolysis
was also examined by two-dimensional chromato-
graphy, developing first with butanol–propanol–
0·1 N-NH$_3$, then treating with acid at 70°, drying,
and developing in the second direction with
butanol–acetic acid–water (2:1:1, by vol.). Two
spots corresponding in R_F and colour reactions to
2-amino-6-naphthol were present after the second
development, one derived from the 2-acetamido-6-
naphthol spot and the other from that of the un-
known compound. The properties of this latter
compound can be accounted for by the hydro-
lysis and dehydration of 2-acetamido-5:6-dihydro-
5·6-dihydroxynaphthalene. Acid-catalysed dehydra-
tion of cyclic dihydrodiols usually proceeds so
that a hydroxyl group remains on the position
which is normally attacked by electrophilic re-
agents (Badger, 1949). For example 3:4-dihydro-
3:4-dihydroxychlorobenzene yields 4-hydroxy-
chlorobenzene (Smith, Spencer & Williams, 1950)
and 1:2-dihydro-1:2-dihydroxynaphthalene gives 1-
naphthol (Young, 1947), although some 2-naphthol
is also formed (Boyland & Sims, 1953) In the case

under consideration 2-acetamido-6-naphthol would probably be the major product (yielding the amino compound by simultaneous hydrolysis). The glucosiduronic acid spot with R_F 0·2 therefore represents a mixture of 2-acetamido-6-naphthyl glucosiduronic acid and 2-acetamido-5;6-dihydro-5;6-dihydroxynaphthalene glucosiduronic acid. 2-Acetamido-1-naphthyl glucosiduronic acid was not detected in either rat or rabbit urines after injection of 2-naphthylamine or 2-acetamidonaphthalene. In support of this 2-acetamido-1-naphthol was not detected by paper chromatography after the enzyme hydrolysis of any of the glucuronide fractions.

DISCUSSION

Two N-glucosiduronic acid derivatives (2-naphthylamine N-glucosiduronic acid and 2-amino-1-naphthylsulphuric acid N-glucosiduronic acid) have been described as metabolites of 2-naphthylamine by Boyland et al. (1957). Four O-glucosiduronic acid derivatives are now described (three of which are metabolites of 2-naphthylamine) and evidence is given for the presence of a fourth O-glucosiduronic acid metabolite. It has been argued (Boyland, 1956) that only 2-amino-1-naphthyl glucosiduronic acid should be carcinogenic because the urinary β-glucuronidase would liberate 2-amino-1-naphthol from this. Bonser, Bradshaw, Clayson & Jull (1956) have shown that 2-amino-1-naphthol is carcinogenic when introduced into the bladders of mice. Using a similar technique Allen et al. (1957) have shown that 2-amino-1-naphthyl glucosiduronic acid induces bladder cancer in mice but that other metabolites, including 2-naphthylsulphamic acid, 2-naphthylamine N-glucosiduronic acid, 2-amino-6-naphthol and 2-acetamido-6-naphthol, are inactive. The other three of the glucosiduronic acid derivatives which have been isolated are now being tested for carcinogenic activity.

The occurrence of 2-amino-1-naphthyl glucosiduronic acid in urine of rats dosed with 2-naphthylamine is difficult to reconcile with the fact that rats do not develop bladder cancer when dosed with 2-naphthylamine, although rat urine contains much more β-glucuronidase than does human urine (D. C. Williams, personal communication). The β-glucuronidase should liberate the carcinogenic 2-amino-1-naphthol when the urine of rats dosed with 2-naphthylamine is allowed to stand. It is not liberated unless β-glucuronidase is added to this urine. This apparent anomaly is being investigated.

SUMMARY

1. 2-Acetamido-1-naphthyl glucosiduronic acid was isolated from the urine of rabbits dosed with 2-acetamido-1-naphthol.

2. The carcinogenic 2-amino-1-naphthyl glucosiduronic acid was prepared by hydrolysis of 2-acetamido-1-naphthyl glucosiduronic acid.

3. 2-Amino-6-naphthyl glucosiduronic acid was isolated from the urine of rabbits dosed with 2-amino-6-naphthol.

4. 2-Acetamido-6-naphthyl glucosiduronic acid was isolated as the sodium and p-toluidine salts from the urine of rabbits dosed with 2-acetamidonaphthalene and as methyl (2-acetamido 6-naphthyl tri-O-acetylglucosid)uronate from the urine of rabbits dosed with 2-naphthylamine.

5. Evidence is presented that a glucosiduronic acid of 2-acetamido-5;6-dihydro-5;6-dihydroxynaphthalene is excreted when rabbits are dosed with 2-naphthylamine or 2-acetamidonaphthalene.

6. Urine from rats and rabbits injected with 2-naphthylamine has been examined by paper chromatography. Rats and rabbits excrete 2-amino-1-naphthyl glucosiduronic acid. Rabbits excrete 2-acetamido-6-naphthyl glucosiduronic acid, but this compound was not detected in rat urine. Rats excrete 2-amino-6-naphthyl glucosiduronic acid, but rabbits do not. 2-Acetamido-1-naphthyl glucosiduronic acid was not detected in the urine of either species.

7. Allantoin (which gives a red colour with the naphthoresorcinol reagents) was isolated by adsorption on charcoal and elution with methanol.

REFERENCES

Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S. & Watson, J. G. (1957). Brit. J. Cancer (in the Press)

Asher, K. (1910). Biochem. Z. 26, 370.

Badger, G. M. (1949). J. chem. Soc. p. 2497.

Bonser, G. M., Bradshaw, L., Clayson, D. B. & Jull, J. W. (1956). Brit. J. Cancer, 10, 1956.

Booth, J. & Boyland, E. (1957). Biochem. J. 66, 73.

Booth, J., Boyland, E. & Manson, D. (1955). Biochem. J. 60, 62.

Boyland, E. (1956). Bull. Soc. Chim. biol. Paris, 38, 827

Boyland, E. & Manson, D. (1955). Biochem J. 60, ii

Boyland, E., Manson, D. & Orr, S. F. D. (1957). Biochem J. 65, 417.

Boyland, E. & Sims, P. (1953). J. chem. Soc. P. 2966

Corner, E. D. S., Billett, F. S. & Young, L. (1954). Biochem. J. 56, 270.

Corner, E. D. S. & Young, L. (1955). Biochem J. 61, 132

Dalgliesh, C. E. (1952). Biochem J. 52, 3

Dische, Z. (1947). J. biol. Chem. 167, 189.

Liebermann, C. & Jacobson, P. (1882). Liebigs Ann. 211, 36

Smith, J. N., Spencer, B. & Williams, R. T. (1950). Biochem. J. 47, 284

Young, L. (1947). Biochem J. 41, 417.

collected. After two crystallizations from aqueous ethanol *methyl (2-acetamido-6-naphthyl tri-O-acetylglucosid)uronate* was obtained as needles (0·7 g.), m.p. 205–206°, $[\alpha]_D^{23}$ −28·6° in ethanol (c, 0·26) (Found: C, 58·1; H, 5·0; N, 2·7. $C_{25}H_{27}O_{11}N$ requires C, 58·0; H, 5·3; N, 2·7%). Although the original crude solid appeared to contain 2-acetamido-5·6-dihydro-5·6-dihydroxynaphthalene glucosiduronic acid no crystalline triacetyl methyl ester of this could be isolated

Isolation of allantoin

The first and second cold methanol eluates from charcoal columns (see p 277) were combined and the solution was concentrated to 30 ml On cooling, prisms (1·5 g. from 10 l of urine) separated, m p after three crystallizations from water 234–236° (decomp.), alone and mixed with an authentic specimen of allantoin (Found: N, 35·6. Calc for $C_4H_6O_3N_4$; N, 35·4%). The compound had an R_F of 0·1 in butanol–propanol–0·1 N-NH₄, identical with that of allantoin. The spot gave a yellow colour with Ehrlich's reagent and a green colour with sodium hypochlorite (5% in water) either spraying with phenol (5% in 95% ethanol). On heating either the isolated substance or the authentic allantoin in 5 N-HCl with naphthoresorcinol at room temperature it gave a cherry-red colour, extractable by ether Asher (1910) described this colour reaction of allantoin and the adsorption of allantoin by charcoal. Corner & Young (1955) described the formation of a reddish purple colour from an unidentified constituent with naphthoresorcinol during the estimation of glucuronic acid in rat urine, but the colour did not interfere with the estimations. It is possible that allantoin was a

The O-glucosiduronic acids formed in the metabolism of 2-naphthylamine

After rats and rabbits had been dosed with 2-naphthylamine the urine of each species was examined by paper chromatography for the presence of glucosiduronic acids. 2-Amino-1-naphthyl glucosiduronic acid was detected in the urine of both species, but was more abundant in that of the rat. Rat urine (2 ml), diluted with an equal volume of 0 2 M-acetate buffer (pH 4·5), was incubated with calf-spleen β-glucuronidase at 37° for 2 days. 2-Amino-1-naphthol was liberated and identified by the ammonia–benzene test. A control urine sample gave no colour reaction to this test. A fraction containing the compound could be obtained by precipitation of the urine of rats or rabbits by basic lead acetate.

2-Amino-6-naphthyl glucosiduronic acid was detected in rat urine but not in rabbit urine The compound was precipitated from rat urine by basic lead acetate. After decomposition of the lead salt by hydrogen sulphide a sample of the glucosiduronic acid fraction so obtained gave 2-amino-6-naphthol on incubation with calf spleen β glucuronidase Rabbit urine, but not rat urine, contained a compound (R_F 0·2) which was non fluorescent and which was not diazotizable until after treatment

with acid at 70°. It corresponded in its reactions to 2-acetamido-6-naphthyl glucosiduronic acid. Paper chromatography of the urine after it had been heated for 15 min. with an equal volume of 5 N-hydrochloric acid gave a new spot which corresponded in R_F and colour reactions to 2-amino-6-naphthyl glucosiduronic acid. Although only one glucosiduronic acid appeared on hydrolysis there are two possibilities for the structure of its precursor. Both 2-acetamido-6-naphthyl glucosiduronic acid and 2-acetamido-5·6-dihydro-5·6-dihydroxynaphthalene glucosiduronic acid would give the amino compound on acid treatment The glucosiduronic acid fraction of rabbit urine (50 ml.) was prepared by precipitation with basic lead acetate. After decomposition of the lead salt by hydrogen sulphide excess of the latter was removed by a current of air. The solution was adjusted to pH 6·5 and continuously extracted with ether for 18 hr. and then evaporated to 5 ml. in a desiccator at room temperature. After dilution with an equal volume of acetate buffer (pH 6·0) a sample of the solution was incubated with bacterial β-glucuronidase Paper chromatography showed that two new spots were present. One corresponded to 2-acetamido-6-naphthol in R_F (0·92) and colour reactions. The other spot (R_F 0 81) was not fluorescent, did not react with diazotized sulphanilic acid, could not be diazotized and coupled with hexylresorcinol, but after treatment with acid at 70° the material in the spot could be diazotized (green colour with nitrous acid) and coupled with hexylresorcinol (red colour), and coupled with diazotized sulphanilic acid (mauve colour)—reactions identical with those of 2-amino-6-naphthol.

The material obtained by enzymic hydrolysis was also examined by two-dimensional chromatography, developing first with butanol–propanol–0 1 N-NH₄, then treating with acid at 70°, drying, and developing in the second direction with butanol–acetic acid–water (2.1 : 1, by vol.) Two spots corresponding in R_F and colour reactions to 2-amino-6-naphthol were present after the second development, one derived from the 2-acetamido-6-naphthol spot and the other from that of the unknown compound The properties of this latter compound could be accounted for by the hydrolysis and dehydration of 2-acetamido-5·6-dihydro-5·6-dihydroxynaphthalene. Acid-catalysed dehydration of cyclic dihydrodiols usually proceeds so that a hydroxyl group remains on the position which is normally attacked by electrophilic reagents (Badger, 1949). For example 3:4-dihydroxy-3:4-dihydroxychlorobenzene yields 4-hydroxychlorobenzene (Smith, Spencer & Williams, 1950) and 1:2-dihydro-1:2-dihydroxynaphthalene gives 1-naphthol (Young, 1947), although some 2 naphthol is also formed (Boyland & Sims, 1953). In the case

after the urines had been heated with acid, and Sato *et al.* (1956) found that the incubation of 1:2-dihydroxynaphthalene and ^{35}S-labelled sulphate with liver slices also yielded the new metabolite. All the above workers found that the metabolite was also produced when rats were treated with *trans*-1:2-dihydronaphthalene-1:2-diol. Corner & Young (1954) showed that the new metabolite, which must be either 2-hydroxy-1-naphthyl sulphate or 1-hydroxy-2-naphthyl sulphate, was present in the urine of guinea pigs, mice and rabbits dosed with naphthalene and they detected free 1:2-dihydroxynaphthalene in the guinea-pig urine.

The present work describes the isolation of 2-hydroxy-1-naphthyl sulphate from the urine of rabbits dosed with naphthalene, which was identical with the synthetic ester prepared by the Elbs persulphate oxidation of 2-naphthol. Desai & Sethna (1951) isolated 1:2-dihydroxynaphthalene from this oxidation.

EXPERIMENTAL

1:2-Dihydroxynaphthalene. This was prepared by the reduction of 1:2-naphthaquinone with sodium dithionite by the method of Fieser & Fieser (1939). The crude product was purified by distillation in a microsublimation apparatus (180° at 12 mm.), to yield 1:2-dihydroxynaphthalene in yellowish needles, m.p. 102°. The diacetate separated from ethanol in prisms, m.p. 109° (Found. C, 68 8; H, 5·2 Calc. for $C_{14}H_{12}O_4$. C, 68·8; H, 4 95%). Fieser & Peters (1931) recorded m.p. 109 5° for this compound.

Potassium and sodium 2-hydroxy-1-naphthyl sulphate. 2-Naphthol (10 g) and KOH (10 g.) in water (100 ml.) were cooled to 0° and to a saturated aqueous solution of $K_2S_2O_8$ (20 g.) was added during 8 hr. with continuous stirring The mixture was kept overnight at room temperature, the dark solid was filtered off and discarded, and the filtrate was adjusted to pH 4 with acetic acid. The solution was washed with ether (3 × 100 ml.) to remove unchanged 2-naphthol, and passed through a bed of charcoal (100 g., activated, British Drug Houses Ltd) supported in a glass chromatography tube The bed was washed with water (2 l.) to remove inorganic sulphate, with aqueous $2N-NH_3$ soln. (1 l.), which removed coloured materials, and finally with methanol (1·5 l) to elute the sulphuric ester. The methanolic eluate was evaporated to dryness under reduced pressure and the residue was recrystallized from aqueous ethanol to yield *potassium 2-hydroxy-1-naphthyl sulphate* (3 4 g.) in elongated plates (Found: C, 43·2; H, 2 8; S, 10·9; K, 13 9 $C_{10}H_7O_4SK$ requires C, 43 15; H, 2 5, S, 11·5, K, 14 05%).

When the potassium salt (1 g.) in $5N-HCl$ (5 ml.) was heated to 100° for 30 min. and cooled to 0°, 1:2-dihydroxynaphthalene (380 mg.) separated in glistening plates (m.p. 58–59°), which darkened on exposure to air. The crystals were dried at 100° at 0·2 mm. and recrystallized from light petroleum (b.p. 60–80°) in needles, m.p. and mixed m.p. 98–100°. The diacetate formed prisms from ethanol, m.p. and mixed m.p. 109°. The mother liquors from the hydrolysis were shown, by the use of $BaCl_2$, to contain inorganic sulphate.

The potassium salt (2 g.) was dissolved in $5N-NaOH$ (10 ml.) and ethanol (50 ml.) was added. The solution was warmed for a few minutes and cooled to 0°, when a substance which appeared to be the disodium salt of 2-hydroxy 1 naphthyl sulphate separated, which formed silky needles from aqueous ethanol. This substance was hygroscopic and proved unsuitable for analysis. A concentrated aqueous solution of the salt was therefore acidified to pH 2 with conc. HCl, when *sodium 2-hydroxy-1-naphthyl sulphate* separated. From methanol-ether it formed plates (Found. C, 45 6; H, 2 9; S, 11 7; Na, 9 0 $C_{10}H_7O_5SNa$ requires C, 45 8; H, 2 7; S, 12 2; Na, 8 8%) The sodium salt was hydrolysed with $5N-HCl$ and the product was purified as before to yield 1:2-dihydroxynaphthalene, m p. and mixed m p. 100–101°.

Properties of 2-hydroxy-1-naphthyl sulphate on paper chromatograms. On descending chromatograms on Whatman no. 1 chromatography papers, the ester had R_F 0 48 when the papers were developed with n-butanol saturated with $2N-NH_3$ soln., R_F 0·45 with the organic phase of a mixture of n-butanol-ethanol-water (17.3.20, by vol.) (Solomon, 1953) and R_F 0 26 with the organic phase of the ...

... bright-violet fluorescent spot when the papers were examined under a Hanovia Chromatolite ultraviolet lamp fitted with a Chance OX7 filter. When the chromatograms were sprayed with diazotized *p*-nitroaniline (0 2% in ... ... / ... HCl),followed in each case by aqueous 10% Na_2CO_3 the ester developed little or no colour within 4 hr , but later pale-brown colours appeared. When the papers were sprayed with $2N-HCl$ and heated in an oven to 70° for 20 min., the ester gave immediate purple colours (characteristic of free 1,2-dihydroxynaphthalene) with each of the reagents.

Dosing of animals and collection of urine. Four rabbits (body weight approximately 2 kg), maintained on oats, rat cake, bread and water, were each injected intraperitoneally on four consecutive days with a solution of naphthalene in arachis oil (5 ml. of 20%, w/v). The animals were housed in metabolism cages designed to allow the collection of urine separately from faeces. The urines were collected up to the sixth day and were stored at 0°.

Isolation experiments. The urine was centrifuged to remove solid matter, acidified (pH 4) with acetic acid and washed several times with ether. It was then passed through a bed of activated charcoal (500 g), which was afterwards washed with water (4 l) to remove urea and inorganic salts, and then with aqueous $2N-NH_3$ soln. (1·5 l.). The naphthalene metabolites were finally eluted with methanol (2 l.), and the eluate was evaporated to dryness under reduced pressure to a brown gum. ... ... of ... d ... o ... h form a slurry, which was poured into a glass column (leng h 100 cm , diameter 8 cm) fitted with a sintered disk as a ...

was passed through the column at the rate of 100 ...

Metabolism of Polycyclic Compounds

11. THE CONVERSION OF NAPHTHALENE INTO 2-HYDROXY-1-NAPHTHYL SULPHATE IN THE RABBIT*

BY E. BOYLAND AND P. SIMS

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By the use of paper-chromatographic analysis, Corner & Young (1954, 1955), Sato, Yamada, Suzuki, Fukuyama & Yoshikawa (1956) and Solomon (1955) detected the presence of a metabolite of naphthalene in the urine of rats dosed with naphthalene, which gave the colour reactions of

* Part 10: Boyland & Solomon (1956).

1:2-dihydroxynaphthalene after the chromatograms had been treated with mineral acid. By injecting ³⁵S-labelled sodium sulphate into the dosed animals, the first two groups of workers were able to show by the use of radiochromatograms that the metabolite was probably a sulphuric ester of 1:2-dihydroxynaphthalene. Furthermore, Corner & Young (1955) detected 1:2-dihydroxynaphthalene

The Oxidation of Aromatic Amines. Part V.* Oxidation by Perphosphoric Acids.

By E. BOYLAND and D. MANSON.

Aromatic amines are oxidised by permonophosphoric acid or a mixture of peracetic and phosphoric acid. Aniline is oxidised to *p*-aminophenol and *p*-aminophenyl dihydrogen phosphate. 2-naphthylamine to 2-amino-1-naphthol and 2-amino-1-naphthyl dihydrogen phosphate; but *NN*-dimethyl-2-naphthylamine gives its *N*-oxide, and anthranilic acid yields azoxybenzene-2.2'-dicarboxylic and 5-hydroxyanthranilic acid. The oxidation to phenolic derivatives does not proceed in neutral or alkaline solution, or in the absence of acetone or some other carbonyl derivatives.

AROMATIC amines are oxidised by mammals to aminophenols which are excreted as sulphuric esters or glucuronides The possibility that the oxidations were oxidative phosphorylations, yielding aminophenyl phosphoric esters, has been examined[1] but no evidence for the presence of 2-amino-1-naphthyl dihydrogen phosphate could be found on biological oxidation of 2-naphthylamine. The oxidation of aromatic amines with perphosphoric acid has been studied (*a*) because it might be analogous to the biological oxidation, (*b*) as a method of preparation of the aminophenyl phosphates as reference compounds in connection with the biological oxidation of amines, and (*c*) for comparison with oxidation by persulphate.[2] Mammals excrete phenols as sulphuric esters and, surprisingly, not as phosphoric esters although much more phosphate than sulphate is excreted

It was expected that phosphoric esters of aminophenols would be formed by oxidation with alkaline perdiphosphate because oxidation with alkaline neutral or acidified persulphate yields *o*-aminophenyl sulphates as the main products.[2] Neither potassium nor ammonium perdiphosphate oxidised 2-naphthylamine to phosphoric esters. The production of both 2-amino-1-naphthol and its phosphoric ester by oxidation by permonophosphoric acid in the presence of acetone was seen in preliminary experiments with the mixed acids formed by the reaction of pyrophosphoric acid with hydrogen peroxide.[3] When the mixed acids were added to 2-naphthylamine in 50% aqueous acetone containing an excess of alkali, no aminonaphthol derivatives were formed at room temperature or at the boiling point If the alkali was omitted, however, 2-amino-1-naphthol and 2-amino-1-naphthyl phosphate were produced, but only if acetone was present. As perdiphosphoric acid is stable in alkali but decomposed in acid solution, while permonophosphoric acid is stable in acid only,[3] these results suggest that the oxidation to aminophenols requires permonophosphoric acid.

Permonophosphoric acid free from perdiphosphoric acid can be prepared by treating hydrogen peroxide with phosphoric oxide in acetonitrile.[4] This material in acetone solution oxidised 2-naphthylamine to 2-amino-1-naphthol and to 2-amino-1-naphthyl dihydrogen phosphate. In aqueous acetone the yield of phosphate was greatly reduced, and in the absence of acetone or other ketones or in neutral solution no phosphate was formed The yield was not significantly altered by changing the rate of addition of the per-acid or by varying the temperature of the reaction from −5° to the boiling point of the mixtures. Oxidation with an excess of permonophosphoric acid gave 2-carboxycinnamic acid. 2-Amino-1-naphthyl dihydrogen phosphate was also readily prepared by oxidation of 2-naphthylamine with peracetic acid in the presence of phosphoric acid and acetone. 2-Amino-1-naphthol, also produced in the oxidations, was not isolated but it was detected

The gum containing the metabolites was dissolved in a little water and the solution was absorbed in cellulose powder (5 g.) which was packed in a layer on top of the cellulose in the column. The solvent mixture was passed through the column at 50 ml./hr., when a broad band which had a violet fluorescence under ultraviolet light was seen to form. The eluate was collected in 100 ml. fractions, which were separately evaporated to dryness under reduced pressure and examined by means of paper chromatography with the solvent systems already mentioned. The earlier fractions contained mainly trans-1,2-dihydronaphthalene-1,2-diol and were discarded. Later fractions, which corresponded to the elution of the fluorescent band, were evaporated to form light-brown gums or partially crystalline solids, all of which contained 1-naphthyl sulphate, together with the new metabolite. These gums were combined and dissolved in water (5 ml.) and 5 N-NaOH (5 ml.) was added, followed by ethanol (50 ml.). The mixture was warmed until crystals began to separate, cooled to 0° and filtered. The filtrate was evaporated down to half volume on the steam bath and cooled to 0°, when more crystals separated. The combined solids were recrystallized from aqueous ethanol to yield a substance which was probably the disodium salt of 2-hydroxy-1-naphthyl sulphate as silky needles (980 mg., after drying at 100° and 0·2 mm.), corresponding to about 2% of the naphthalene administered, which had an infrared spectrum (measured, as a mull in liquid paraffin, between 5 and 15 μ) identical with that of the substance obtained from the oxidation of 2-naphthol. It was converted as before into sodium 2-hydroxy-1-naphthyl sulphate (420 mg.), which formed plates from methanol–ether (Found: C, 45·4, H, 2·7; S, 12·0; Na, 9·0. Calc. for $C_{10}H_7O_5SNa$. C, 45·8, H, 2·7; S, 12·2; Na, 8·8%). The infrared spectrum (measured, as a mull in liquid paraffin, between 5 and 15 μ) was identical with that of the synthetic sodium salt, and the two salts had identical properties on paper chromatograms. When urines of the treated animals were examined on paper chromatograms with the solvent systems, the ester had somewhat higher R_F values than those obtained when the pure compounds were run (cf. Solomon, 1955; Corner & Young, 1954, 1955).

The ester was hydrolysed with 5 N-HCl and the product was purified as before to yield 1,2-dihydroxynaphthalene, m.p. and ·mixed m.p. 101–102°. The diacetate formed prisms from ethanol, m.p. and mixed m.p. 108–109°. The mother liquors from the hydrolysis contained inorganic sulphate.

DISCUSSION

The structure of the new metabolite follows directly from its method of synthesis and from the fact that it gives 1,2-dihydroxynaphthalene and sulphuric acid on acid hydrolysis. The present work throws no light on the method of formation of the ester in the

body but the work of Corner & Young (1955) and of Sato et al. (1956) indicates that 1:2-dihydronaphthalene-1:2-diol is an intermediate in its formation.

The metabolic pathway of naphthalene therefore involves oxidation to 1 : 2-dihydronaphthalene-1 : 2-diol (II). The diol (II) is then dehydrogenated to 1:2-dihydroxynaphthalene (III) which is then conjugated to yield the sulphuric ester (IV).

SUMMARY

1. The isolation of 2-hydroxy-1-naphthyl sulphate from the urine of rabbits dosed with naphthalene is described.

2. The ester was synthesized by the persulphate oxidation of 2-naphthol.

REFERENCES

Boyland, E. & Solomon, J. B. (1956). Biochem. J. 63, 679

Corner, E. D. S. & Young, L. (1954). Biochem. J. 58, 647

Corner, E. D. S. & Young, L. (1955). Biochem. J 61, 132

Desai, R. B. & Sethna, S. (1951). J. Indian chem. Soc. 28, 213.

Fieser, L. F. & Fieser, M. (1939). J. Amer. chem Soc. 61, 596.

Fieser, L. F. & Peters, M. A. (1931) J. Amer. chem. Soc. 53, 793.

Sato, T., Yamada, M., Suzuki, T., Fukuyama, T. & Yoshikawa, H. (1956). J. Biochem., Tokyo, 43, 25.

Solomon, J. B. (1955). Ph D Thesis: University of London

to occur only in the presence of a carbonyl-compound. Robertson and Waters,[8] and Criegee,[9] have suggested that the oxidation of oxopolymethylenes to lactones by permono-sulphuric acid (Baeyer and Villiger reaction [10]) might proceed by way of an addition compound between the carbonyl compound and the per-acid. It is also known that aldehydes and ketones react with hydrogen peroxide or hydroperoxides to give hydroxyalkyl peroxides. Doering and Dorfman [11] investigated the conversion of [^{18}O]benzophenone into phenyl benzoate by perbenzoic acid and found the results to be consistent with an initial addition of the per-acid to the ketone. Similar addition compounds may play a part in the oxidation of amines by permonophosphoric acid in the presence of carbonyl compounds. The oxidation of NN-dimethyl-2-naphthylamine to an amine oxide, and of anthranilic acid to an azoxy-compound, are reactions typical of hydrogen peroxide or organic per-acids.

EXPERIMENTAL

Paper chromatography was carried out by ascending development on Whatman no. 1 paper, with butan-1-ol-acetic acid-water [(a) 2 : 1 : 1 or (b) 4 . 1 : 5 by vol.]

Preparation of Permonophosphoric Acid.—Toennies [4] prepared the per-acid on a small scale It was prepared safely in larger quantities provided the cooling and mixing were carefully controlled. Use of ether as a solvent on this scale (in place of acetonitrile), however, resulted in an explosion. Phosphoric oxide (14·2 g.) was suspended in acetonitrile (30 ml.) at −5°, and hydrogen peroxide (6·8 g. as 7·8 g. of 87% hydrogen peroxide; Laporte Ltd.) and water (0·8 g.) in acetonitrile (10 ml.) were added during 45 min. with stirring, so that the temperature did not rise above 10°. The mixture was kept cold for 1 hr. and then at room temperature overnight; it was stored in a refrigerator and assayed iodometrically before use as described by Toennies.[4] The yield of per-acid was 10·5 g. (46%) and 3 3 g. of hydrogen peroxide remained unchanged. On storage at 5° the per-acid and hydrogen peroxide content fell. 1 ml. of solution, containing initially 0·34 g. of H_3PO_4, contained 0·21 g. of per-acid and 0·02 g. of peroxide after 26 days. Another 1 ml., containing initially 0 25 g. of per-acid and 0 06 g. of peroxide, had after three months in the cold, 0 04 g. and 0 002 g. respectively.

Oxidation of 2-Naphthylamine by Permonophosphoric Acid.—(a) *In acetone* To 2-naphthyl-amine (7 g.) in acetone (150 ml.) permonophosphoric acid (5 g., in acetonitrile) was added dropwise with stirring during 30 min. After a further 30 minutes' stirring, the solution was made alkaline with 2N-sodium hydroxide, and most of the acetone removed under reduced pressure. Water was added and the solution extracted with ether (5 × 100 ml.), acidified with concentrated hydrochloric acid, and extracted with butan-1-ol (5 × 100 ml.). The combined butanol extracts were made alkaline with aqueous ammonia (d 0 88), then evaporated to dryness under reduced pressure, and the residue was dissolved in dilute ammonia solution. The solution was treated with charcoal whilst hot, filtered, and acidified with concentrated hydrochloric acid, 2-amino-1-naphthyl dihydrogen phosphate (1·2 g.) separating. Recrystallisation by dissolution in alkali and precipitation with concentrated hydrochloric acid gave prisms, m p. 247—249° (Found: P, 12 7, N, 5 6. $C_{10}H_{10}O_4PN$ requires P, 13·0; N, 5 9%). Hydrolysis of the ester (0 5 g.) by 5N-sulphuric acid (5 ml.) at 100° for 30 min. yielded, on cooling, 2-amino-1-naphthyl hydrogen sulphate. The N-benzoylbenzoate formed needles (from ethanol), m. p. and mixed m. p. 180° (Found N, 3 9. Calc for $C_{24}H_{17}O_3N$. N, 3·8%). The phosphoric ester gave a brick-red colour after diazotisation and coupling with hexylresorcinol. It gave a positive reaction in Wade and Morgan's phosphate test,[12] but did not respond to Hanes and Isherwood's test,[13] unlike 1-naphthyl dihydrogen phosphate. In solvent system (a) the ester had R_F 0·72.

When ethyl methyl ketone was used as a solvent in the oxidation, the yield of ester from 7 g. of amine was 0 4 g.

The ester reacted immediately as an amine with p-dimethylaminobenzaldehyde to give a yellow colour, and was only slowly hydrolysed by 5N-sulphuric acid at 100°.

(b) *In aqueous acetone.* 2-Naphthylamine (7 g.) was dissolved in acetone (100 ml.) and water (50 ml.), and per-acid (5 g.) was added during 15 min. The mixture was kept overnight and the phosphoric ester (0 1 g.) isolated as in (a). When the mixture was worked up 40 min. after the addition of the per-acid the yield was 0 15 g., when the reaction was carried out at −5° and the mixture worked up after 40 min. it was 0 04 g.; after 1 hour's refluxing, it was 0·1 g.

(c) *Oxidation at pH 6·0.* The reaction was carried out as in (b) but the pH of the per-acid

by the green product formed on addition of ammonia, and was extracted by benzene as a purple solution.[5] 2-Acetamidonaphthalene resisted oxidation by permonophosphoric acid or by a mixture of peracetic and phosphoric acid. NN-Dimethyl-2-naphthylamine with permonophosphoric acid in aqueous acetone gave the amine oxide; Auerbach and Wolffenstein[6] found that hydrogen peroxide had no effect on NN-dimethylnaphthylamine.

Aniline and permonophosphoric acid gave p-aminophenol and p-aminophenyl dihydrogen phosphate in the presence of acetone, but not in acetonitrile or water alone. No o-aminophenol was detected. Aniline and peracetic acid in the presence of phosphoric acid gave p-aminophenyl dihydrogen phosphate (readily isolated as the mono-sodium salt) and some p-aminophenol (not isolated). For chromatographic identification, p-amino-phenyl phosphate was prepared by catalytic reduction of disodium p-nitrophenyl phosphate.

Oxidation of anthranilic acid in aqueous acetone by permonophosphoric acid gave azoxybenzene-2 : 2'-dicarboxylic acid and a small amount of 5-hydroxyanthranilic acid, but no 3-hydroxyanthranilic acid. Oxidation of 1-naphthylamine, benzidine, or 2-amino-fluorene with permonophosphoric acid in acetone gave small amounts of phosphoric esters detected as slow-running amino-compounds on paper chromatograms. No 3 : 3'-di-hydroxybenzidine was detected among the products of the benzidine oxidation.

Different solvents for permonophosphoric acid oxidations were investigated, the formation of 2-amino-1-naphthol and 2-amino-1-naphthyl dihydrogen phosphate being studied. The results are tabulated. Some of the solvents were compared with acetone

Solvents in which no phenolic products were formed		Solvents in which 2-amino-1-naphthol and its phosphate were formed	
Water	Chloroform	Acetone	Acetonylacetone
Acetonitrile	Pyridine	Aqueous acetone (50%)	Diacetyl
Pentyl alcohol (BS696)	Acetic acid	Ethyl methyl ketone	Et acetoacetate
Propan-2-ol	Ethyl acetate	Diethyl ketone	Pyruvic acid (10% aq.)
Ethanol	Diisobutyl ketone	Acetophenone	Formaldehyde (33% aq.) *
Ether	Salicylaldehyde	Benzophenone	Acetaldehyde (50% aq.) †
Dioxan		Acetylacetone	Benzaldehyde †

* Only 2-amino-1-naphthol formed.   † Only the phosphate formed.

in the synthesis of the phosphoric ester on a preparative scale, ethyl methyl ketone gave a lower yield. If N-benzylidene-2-naphthylamine in aqueous suspension or in aceto-nitrile was treated with the per-acid, 2-amino-1-naphthyl phosphate was formed. From these results it appears that carbonyl derivatives facilitate the oxidation.

The formation of compounds between the permonophosphoric acid and acetone was investigated by comparing solutions in acetonitrile and in acetone on paper chromatograms. The acetone solution showed two spots which were not present in the acetonitrile solution: one (R_F 0·5) gave a positive reaction for phosphate ion and for oxidising activity: the other (R_F 0·85) consisted of an oxidising substance which gave no phosphate reaction. Neither of these spots was obtained if acetone was treated with hydrogen peroxide only. If an acetone solution of the per-acid was kept for two days at room temperature the per-acid disappeared, but the products giving the spots described above remained and the solution was still capable of oxidising 2-naphthylamine to 2-amino-1-naphthol and its phosphoric ester.

Horner and Schwenk[7] proposed mechanisms for the reaction of benzoyl peroxide with aniline, methylaniline, and dimethylaniline the postulated primary step is a transfer of one electron from the unshared pair of the nitrogen atom to one of the peroxidic oxygen atoms, resulting in fission of the $^-$O–O$^-$ bond and formation of a radical and a negative ion. A similar mechanism may account for the formation of phenols and phosphoric esters by permonophosphoric acid The per-acid might be split by the transfer of an electron from the amino-nitrogen atom to give hydroxyl or phosphate radicals. The formation of p-aminophenol and 2-amino-1-naphthol and their phosphoric esters seems

the solution extracted with ether. Paper chromatography showed the extract to contain aniline and p-aminophenol. Extraction of the aqueous layer, adjusted to pH 5·0 by 2N-hydrochloric acid, with butan-1-ol removed more p-aminophenol. During this last extraction a solid (2·1 g.) crystallised from the aqueous layer, and was collected. The ester was isolated as sodium p-aminophenyl hydrogen phosphate (Found: P, 14·3; N, 6·3, Na, 11·5. $C_6H_7O_4NPNa$ requires P, 14·7, N, 6·6, Na, 10·9%). Hydrolysis of the ester with 5N-hydrochloric acid at 100° for 30 min. and benzoylation of the product yielded p-benzamidophenyl benzoate, m. p. and mixed m. p. 230—232° (Found· N, 4·6. Calc. for $C_{20}H_{15}O_3N$: N, 4·4%). p-Amino-phenyl phosphate gave colour·tests like those of 2-amino-1-naphthyl phosphate

Disodium p-Aminophenyl Phosphate formed by the Reduction of the Nitro-compound.—Disodium p-nitrophenyl phosphate (0·5 g.) in 50% aqueous ethanol (50 ml.) was reduced with hydrogen and Adams catalyst (0·1 g.) at room temperature and pressure. After filtration, the solution was evaporated to dryness and the residue crystallised from aqueous ethanol (approx 50%), to yield *disodium p-aminophenyl phosphate* as needles (0·15 g., 30%) (Found: P, 13·1, N, 5·8 $C_6H_6O_4NPNa_2$ requires P, 13·3, N, 6·0%).

Oxidation of Anthranilic Acid.—Permonophosphoric acid (10 g., in acetonitrile) was added during 30 min. to anthranilic acid (14 g.) in acetone (50 ml.) and water (50 ml.). After 4 hours' stirring and storage overnight the solution was neutralised and evaporated to small volume The solution was adjusted to pH 4·0 and continuously extracted with ether for 24 hr. Yellow prisms crystallised from the ether extract and, after recrystallisation from alcohol, had m. p. 245—246° (1·0 g.) Paper chromatography of the ether extracts in solvent system (b) showed the presence of anthranilic acid (R_F 0·05, yellow colour after diazotisation and coupling with hexylresorcinol) and 5-hydroxyanthranilic acid (R_F 0·6, red colour with above reagent) No 3-hydroxyanthranilic acid appeared to be present (the authentic compound had R_F 0·9 and formed a pale yellow colour with the above reagents) Acidification of the aqueous layer to pH 1·0, followed by extraction with ether, gave more (0·25 g.) of the compound, m. p. 245°, which was acidic, did not diazotise, couple with hexylresorcinol, or give a colour with diazotised sulphanilic acid The compound gave no m. p. depression with azoxybenzene-2 : 2'-dicarboxylic acid [16] (Found C, 58·8, H, 3·5; N, 10·15 Calc for $C_{14}H_{10}O_5N_2$: C, 58·7; H, 3·5, N, 9·8%) Examination of the aqueous fraction by paper chromatography revealed no diazotisable compounds

Attempted Oxidation of 2-Naphthylamine with Alkaline Perdiphosphate.—2-Naphthylamine (0·5 g.) was treated with perdiphosphate (1 equiv.) and the mixture examined by paper chromatography in solvent system (a). No 2-amino-1-naphthyl phosphate was formed when either potassium or ammonium perdiphosphate under the following conditions: (1) neutral aqueous solution, at room temperature up to 10 days; (2) aqueous alkaline solutions at room temperature, or at 100° for 4 hr., (3) alkaline 50% acetone for up to 10 days at room temperature Reaction in the presence of added phosphoric acid gave no phosphoric esters unless acetone was present However, on a preparative scale (6·3 g. of amine) the use of this method at room temperature gave only a few mg. of phosphoric ester. Experiments with aniline gave similar results

Paper Chromatography of Acetone and Acetonitrile Solutions of Permonophosphoric Acid.—Solutions (10%) of permonophosphoric acid in acetonitrile or acetone were kept for 2 hr at room temperature and samples of each applied to chromatography paper The spots were neutralised with ammonia vapour, and the chromatograms developed in solvent system (a) for 16 hr Phosphates were detected by Hanes and Isherwood's method [13] (ascorbic acid as the reducing agent [17]) Oxidising substances were detected by a 5% aqueous potassium iodide spray Permonophosphoric acid had R_f 0·25 and phosphoric acid R_F 0·35 If the acetone solution was kept for 48 hr at room temperature only the oxidising spots at R_F 0·5 and 0·85 and phosphoric acid were detectable

The effects of solvents on the oxidation of 2-naphthylamine were studied by dissolving the

solution was adjusted to 6·0 by 2N-sodium hydroxide. Titration of an aliquot part indicated no loss of per-acid by this treatment. No phosphoric ester was isolated, although paper chromatography indicated that a trace was present

(d) Use of excess of per-acid in aqueous acetone. 2-Naphthylamine (5 g.) in acetone (140 ml) and water (70 ml.) was treated with per-acid (12 g) and after 1 hr the mixture extracted as in (a). The product (1·5 g.), isolated by the acidification of an aqueous solution of the residue from the butan-1-ol extract, had m p. 190—192° after recrystallisation from aqueous ethanol. It was acidic, did not diazotise or couple with hexylresorcinol, and contained no nitrogen, or phosphorus ·· ·· ···· ·· ··· ·· ···· ·· with 2-carboxycinnamic acid (Found. C, 62·6, H, 4·4 Calc. ·· ·· ·· ·· As described by Titley [14] the melt solidified on further hea·· ·· ·· at a lower temperature (140—144°, Titley reports 151°). The diamide, prepared by treatment with thionyl chloride followed by reaction with ammonia, formed needles, m p and mixed m p. 201—202° (Found. N, 14 9 Calc for $C_{10}H_{10}O_2N_2$: N, 14·7%).

(e) Oxidation in acetonitrile 2-Naphthylamine (7 g) was treated in acetonitrile (60 ml) with the per-acid (4 g.) during 30 min Working up as before yielded only 2-carboxycinnamic acid (0 05 g) from the butan-1-ol fraction Chromatography of this and the original reaction mixture revealed no 2-amino-1-naphthyl dihydrogen phosphate.

Oxidation of 2-Naphthylamine by Peracetic Acid in the Presence of Phosphoric Acid —
2-Naphthylamine (7 g) and phosphoric acid (9 8 g) were dissolved in acetone (150 ml) 40% Peracetic acid (10 ml) was added with stirring during 30 min. Stirring was continued for a further 1·5 hr , then the mixture was made alkaline with 2N-sodium hydroxide, and most of the acetone removed under reduced pressure After dilution with water, the solution was extracted several times with ether, and the aqueous layer acidified, to yield the phosphoric ester (3·5 g), m p and mixed m p 242—244° (decomp.) (N-benzoylbenzoate, m p 180°)

Oxidation of NN-Dimethyl-2-naphthylamine —The amine [13] (5 g) in acetone (100 ml) and water (50 ml.) was treated with the per-acid (3·0 g) during 20 min and stirred for a further 1·5 hr. A crystalline precipitate (2 2 g) was filtered off, and the filtrate neutralised and evaporated to small volume. The aqueous solution was extracted with ether (from which 0 9 g of the amine was recovered) and, after acidification with concentrated hydrochloric acid, with butan-1-ol The butanol extract was neutralised with ammonia and evaporated to dryness. From an aqueous solution of the residue, 0 5 g of a substance, m p. 160—163°, crystallised Recrystallisation of the combined precipitates from 70% aqueous acetone gave plates, m p 167—169° The product was acidic and contained phosphate ion, but was not precipitated on addition of alkali (the amine is not soluble in water) Analysis indicated that the compound was the oxide phosphate (Found · P, 10 8, 10 7, N, 4 9 $C_{12}H_{13}ON,H_3PO_4$ requires P, 10 9; N, 4·9%) The oxide (0 5 g) was kept in concentrated hydrochloric acid with stannous chloride (0·5 g) overnight at room temperature and then made alkaline and extracted with ether Evaporation of the extract and crystallisation of the residue from aqueous ethanol yielded NN-dimethyl-2-naphthylamine, m p and mixed m p. 44—45° (Found C, 83·9, H, 8 1, N, 8 05 Calc for $C_{12}H_{13}N$· C, 84·2, H, 7·7, N, 8·2%).

Oxidation of Aniline by Permonophosphoric Acid —Permonophosphoric acid (10 g in acetonitrile) was added during 30 min with cooling and stirring to aniline (15 g) in acetone (200 ml) After a further 1·5 hr the solution was neutralised with 2N-sodium hydroxide and evaporated to small volume under reduced pressure After dilution with water and adjustment to pH 5 0, the solution was extracted with ether, then with butan-1-ol and again with this solvent at pH 2 0 The ether extracts contained p-aminophenol and evaporation of the butanol extracts and benzoylation of the residue in pyridine with benzoyl chloride yielded p-benzamidophenyl benzoate (10 mg), m p and mixed m p 230—232° Paper chromatography showed that the butanol extracts contained no p-aminophenyl phosphate The ester was identified in the aqueous layer by comparison on paper chromatography with a known specimen [R_F 0 35 in solvent system (a)] The spot gave a yellow colour after diazotisation and coupling with hexylresorcinol Two other spots with the same colour reaction and with R_F respectively 0 56 and 0 16 were present but were not identified. p-Aminophenyl phosphate was not isolated

Oxidation of Aniline by Peracetic Acid in the Presence of Phosphoric Acid —40% Peracetic acid (38 ml) was added during 45 min with cooling to aniline (18 6 g) and phosphoric acid (39 2 g) in acetone (350 ml) Water (50 ml) was added and the mixture neutralised with 2N-sodium hydroxide and evaporated to small volume More water (250 ml) was added and

Tryptophan Metabolism in Patients with Carcinoid and Other Tumours

BY

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The characteristic biochemical abnormality of argentaffinomas is the conversion of tryptophan to 5-hydroxytryptamine or serotonin. Many bacteria convert tryptophan into indole

pellagra-like symptoms.

The major metabolic pathway of tryptophan in normal mammals leads through kynurenine, 3-hydroxykynurenine 3-hydroxyanthranilic acid to nicotinic acid. We have been particularly interested in this process because 3-hydroxyanthranilic acid is carcinogenic when implanted in the bladders of mice and it may be a cause of cancer of the bladder in man (Allen *et al*, 1957) A subsidiary metabolic route involving 3-hydroxykynurenine leads to formation of xanthurenic acid—a substance which is said to induce hyperglycæmia and may be important in diabetes.

Our own approach to the carcinoid problem has been through interest in those tryptophan metabolites which may be of importance in cancer of the bladder. We (Boyland *et al*, 1956) have developed a method in which some 16 tryptophan metabolites were separated on paper chromatograms and their amounts estimated by the measurement of the areas of the spots produced by the different substances.

In most of the patients twenty-four-hour specimens of urine, collected under normal

are more easily seen.

The method applied to 3 patients with carcinoids gave results similar to those found by other workers. The urine of these patients did not contain abnormal amounts of any of the other possible tryptophan metabolites including anthranilic acid, 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid, 3-hydroxyanthranilic acid sulphuric ester, kynurenine, 3-hydroxykynurenine, tryptamine, and indoxyl sulphate.

The same tryptophan metabolites have been determined in the urine of patients with tumours of different sites. Patients with bladder cancer excrete increased amounts of 3-hydroxyanthranilic acid and 3-hydroxykynurenine (Boyland and Williams, 1956). In of the vocal

of the breast or by subjects free of malignant disease.

REFERENCES

ALLEN, M. J., BOYLAND, E., DUKES, C. E., HORNING, E S., and WATSON, G (1957) *Brit. J. Cancer* (in press).

HARRISON, M.
R. B (1957) *Lancet*, II, 23

'amine (0 5 g) in the solvent (15 ml.), and adding 0·3 g. of permonophosphoric acid. The mixtures were examined by paper chromatography.
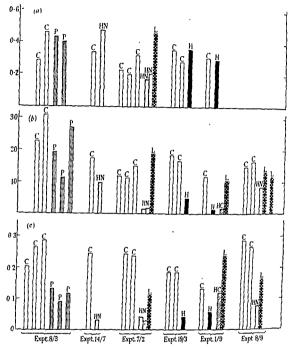
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        FULHAM ROAD, LONDON, S W 3   [Received, June 12th, 1957]

[1] Boyland and Manson, Biochem J., 1955, 60, 11.
[2] Boyland, Manson, and Sims, J , 1953, 3623
[3] Schmidlin and Massini, Ber , 1910, 43, 91
[4] Toennies, J Amer Chem Soc , 1937, 59, 555.
[5] Liebermann and Jacobson, Annalen, 1882, 211, 36
[6] Auerbach and Wolffenstein, Ber , 1901, 34, 2411.
[7] Hermann and Schmidt, Amer Chem 1010, 61, 111
[8]
[9]
[10]   4,   ..; 858
[11]   75, 5595
[12]   ..   :
[13]   : :
[14] Titley, J , 1928, 2571.
[15] Billman, Radike, and Munday, J. Amer Chem Soc , 1942, 64, 2977.
[16] Heller, Ber , 1908, 41, 2690
[17] Rouser and Neuman, Fed. Proc., 1952, 11, 278.

FIG. 1a.—Adenosine deaminase levels, expressed as μ moles inosine
decomposed per min per g. of tissue.
FIG. 1b.—Nucleoside phosphorylase levels, expressed as μ moles
adenosine decomposed per min. per g. of tissue.
FIG. 1c.—Xanthine oxidase levels, expressed as μ moles xanthine
decomposed per min. per g. of tissue.

The following abbreviations are used for the different types of liver tissue :

Bars in direct apposition represent different tissue samples from the same rat.

ADENOSINE DEAMINASE, NUCLEOSIDE PHOSPHORYLASE AND XANTHINE OXIDASE IN LIVER TUMOURS

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ENZYMES concerned in the catabolism of nucleic acids have been little studied in liver tumours, and especially in tumours which, from careful histological examination, could be regarded as hepatomas and could, therefore, reasonably be compared with normal liver. In normal liver the degradation of adenosine can proceed by the following pathway (Christman, 1952) :

$$
\begin{array}{c}
\textit{adenosine} \\
\downarrow \text{ adenosine deaminase} \\
\textit{inosine} \\
\downarrow \text{ nucleoside phosphorylase} \\
\textit{hypoxanthine} \\
\downarrow \text{ xanthine oxidase} \\
\textit{xanthine} \\
\downarrow \text{ xanthine oxidase} \\
\textit{uric acid}
\end{array}
$$

Changes in these enzymes in azo-dye carcinogenesis have now been investigated.

EXPERIMENTAL

The tissue fractions now analyzed were those prepared in a previous study (Reid and O'Neal, 1956), to which reference should be made for details of the procedures employed. The fractions were, in general, derived from the livers of albino rats which had been fed 3'-methyl-4-dimethylaminoazobenzene ; but one experiment (" 14/7 ") was carried out with a hepatoma arising in a rat of the " August " strain which had been fed *p*-dimethylaminophenylazo-2-naphthalene. As indicated in Fig. 1, experimental rats were always studied simultaneously with controls which had been fed on the same diet but with omission of the carcinogen.

The " pre-cancerous liver " studied in some experiments was obtained from rats which had been fed the azo-dye for only 4 weeks. Tissue from rats which had been fed azo-dye for a prolonged period was classified into the following categories, with the kind collaboration of Dr. R. Daoust : *hepatoma with little*

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main pathological change observed in the livers studied by these authors was bile-duct proliferation, and that the tumours which were eventually obtained (but not studied biochemically) were of bile-duct origin. Here it may be noted that experiments in this Institute (Lewin, Bergel, Bray, Haddow and Lewin, 1957) have shown a decrease in xanthine oxidase in spontaneous mammary tumours, similar to that in the hepatomas now studied. Moreover, rabbit liver carcinomas showed abnormally low oxygen uptake with xanthine as subtrate, although " xanthine dehydrogenase " (assayed by the use of methylene blue) was undiminished (Korotkoruchko, 1953).

The possibility that xanthine oxidase is a " key " enzyme, governing the rate of purine catabolism, has been discussed by Bergel, Bray, Haddow and Lewin (1957), and also by Reid and Stevens (1958) who adduced supporting evidence from hormonal studies (Reid, O'Neal and Lewin, 1956) but who quoted some contrary evidence. Thus, Bass, Tepperman, Richert and Westerfeld (1950) found that the fall in liver xanthine oxidase produced by protein deprivation or by azo-dye feeding was not accompanied by decreases in the excretion of uric acid and allantoin. Begg (1955) found that when tumour-bearing rats were given a low-protein diet, the usual fall in liver xanthine oxidase did not occur and the excretion of allantoin actually increased ; but he did not regard it as proven that the latter effect was a consequence of the former.

In view of the possibility that uricase might be a limiting enzyme in liver (Reid and Stevens, 1958), it is of interest that a fall in uricase was observed with the hepatomas studied by Lamirande and Allard (1957) and also, in a single determination, with liver from a rat fed 3'-methyl-4-dimethylaminoazobenzene for 28 days (Schneider, Hogeboom, Shelton and Striebich, 1953).

If purine catabolism is indeed decreased in liver tumours, it does not follow that nucleic-acid catabolism is also decreased. There is some evidence that the nucleases which effect the initial hydrolysis of nucleic acids to nucleotides are actually increased in pre-cancerous liver and in primary liver tumours (Allard, 1955 ; Schneider, Hogeboom, Shelton and Striebich, 1953). The fall in nucleoside phosphorylase does not imply a decrease in nucleic-acid catabolism, since this enzyme, of which there is a relatively high level in liver, is probably not a limiting factor in nucleic-acid catabolism (Reid and Stevens, 1958). The decrease in nucleoside phosphorylase might in fact imply a decrease in synthetic rather than in catabolic processes. The level of adenosine deaminase in normal liver is much lower than that of nucleoside phosphorylase, and would be more likely to reflect any decrease in nucleic-acid catabolism ; however the level of adenosine deaminase in hepatomas is normal (as now reported) or even increased (Lamirande and Allard, 1957). There are reports that the level of nucleotidase (nucleoside-2', 3'-phosphatase) is normal in hepatomas (Greenstein, Carter and Luthardt, 1946), and that the level of a possibly different nucleotidase (nucleotide 5'-phosphatase) is decreased (Lamirande and Allard, 1957). The metabolism of nucleic acids and their derivatives in cancerous liver and, indeed, in normal liver clearly warrants further study.

SUMMARY

With hepatomas induced by azo-dye feeding, there are marked decreases in the concentrations of nucleoside phosphorylase and of xanthine oxidase (the latter being also somewhat decreased in pre-cancerous liver) but no changes in

necrosis, hepatoma with marked necrosis, mixed hepatoma and cholangioma, and *liver tissue without tumours* (tumours being present elsewhere in the liver). Since there was liver enlargement after azo-dye feeding, especially if prolonged (Reid and O'Neal, 1956), any decrease in the amount of an enzyme per g. of liver (cf. Fig. 1) does not entail a correspondingly great decrease in the total amount of the enzyme per liver.

As previously described (Reid and O'Neal, 1956), the tissue homogenates were freed from nuclei by centrifugation to give a cytoplasmic fraction, from a portion of which a supernatant fraction was derived by a final centrifugation at 20,000 g for 90 minutes. These two fractions were frozen and stored at $-30°$ until required for assay, this procedure being without adverse effect on the activity of the enzymes studied.

The enzyme assays were performed by differential spectrophotometry (Kalckar, 1947), the activity of each tissue sample being assessed from the rate of change of extinction (determined graphically) at the appropriate wavelength. Adenosine deaminase and nucleoside phosphorylase were assayed at $21-22°$ essentially by the procedures of Schneider and Hogeboom (1952), as used by Reid and Stevens (1958) in another connection ; the latter enzyme was assayed in the presence of an excess of purified xanthine oxidase whereby the hypoxanthine (formed from inosine) was oxidized to uric acid. Xanthine oxidase was assayed at $24°$ as described by Reid, O'Neal and Lewin (1956) ; whole cytoplasmic fractions were used, although most of the cytoplasmic activity is in fact found in the supernatant fraction. The other enzymes were assayed in supernatant fractions, in which virtually all the cytoplasmic activity resides (Schneider and Hogeboom, 1952).

RESULTS

As is shown in Fig. 1a, there were no consistent changes in adenosine deaminase activity. In contrast with adenosine deaminase, nucleoside phosphorylase activity was notably low in hepatomas, this low level being not merely a consequence of necrosis (Fig. 1b). *The activity was not markedly depressed in liver tissue distant from the tumours.*

Xanthine oxidase activity in hepatomas showed decreases remarkably similar to those observed with nucleoside phosphorylase (Fig. 1c). The activities of these two enzymes *in some of the hepatomas were less than one-quarter of those in* the controls studied simultaneously. Xanthine-oxidase activity was also somewhat decreased in pre-cancerous liver.

DISCUSSION

The present findings are in good agreement with observations which were published in abstract form by Lamirande and Allard (1957) after the completion of our work. These authors found that Novikoff hepatoma transplants lacked xanthine oxidase and uricase, and were deficient in nucleotidase (nucleoside 5'-phosphatase), guanase, and nucleoside phosphorylase, in contrast with these enzymes, adenosine deaminase was increased. Westerfeld, Richert and Hillinger (1950) have also reported a marked fall in xanthine oxidase under the "synergistic" influences of 4-dimethylaminoazobenzene and of a low-protein diet ; but with a high-protein diet, as used in the present study, this carcinogen induced only a small decrease in xanthine oxidase. It may, however, be significant that the

Hormones and Liver Cytoplasm

4. RIBONUCLEOTIDES, RIBONUCLEIC ACID SYNTHESIS AND PROTEIN SYNTHESIS AFTER ADRENALECTOMY*

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After adrenalectomy, the supernatant fraction obtained from rat liver by differential centrifuging shows increases in the amount of ribonucleic acid (RNA) (Reid, 1955) and in the incorporation of injected [6.¹⁴C]orotic acid into the RNA (Reid, O'Neal, Stevens & Burnop, 1956). On the other hand, the microsomal fraction shows a decreased incorporation of orotic acid. This decrease is in accordance with the postulated role of microsomal RNA in protein synthesis, the rate of which as judged by the incorporation of injected DL-[1.¹⁴C]-leucine appears to be slightly diminished after adrenalectomy (Reid et al. 1956). No attempt was made in these studies to ascertain whether adrenalectomy affected the precursor 'pool' or the time course of incorporation. It was not therefore conclusively proved that the increase in the amount of RNA in the supernatant fraction is due to increased synthesis as distinct from decreased catabolism or that this RNA has some function unconnected with protein synthesis.

A closer study has now been made in an endeavour to clarify not only these aspects but also the origin, the fate and the possible metabolic diversity of the RNA of the supernatant fraction.

Liver ribonucleotides have also been investigated, to ascertain whether adrenalectomy affects the ribonucleotide pool and, in particular, the pool of the uridine phosphates into which injected orotic acid is rapidly converted (Hurlbert & Potter, 1952, 1954; Schmitz, Hurlbert & Potter, 1954).

Another aspect which has now been studied, as briefly reported elsewhere (Reid & Stevens, 1956), is the sequence after adrenalectomy of the various changes in the metabolism of RNA and of protein.

EXPERIMENTAL

Experimental procedures are described in detail only where these differ from those employed in Part 1 (Reid et al. 1956). As previously, the animals were male rats, wt. about 250 g., which had been fed on a protein-rich diet in restricted amount and which were fasted overnight before autopsy. Adrenalectomized rats were maintained with saline.

Chromatography of ribonucleotides

Each experiment was performed with pooled liver samples (total 10-11 g.) from two rats. In the later experiments, as indicated in Table 1, the rats were killed by decapitation, and the liver was ground to a powder in the frozen state (liquid nitrogen) before extraction with perchloric acid solution, as described by Hurlbert & Potter (1954) and Hurlbert, Schmitz, Brumm & Potter (1954) but without perfusion of the liver.

Analysis of the neutralized extracts was performed by anion-exchange chromatography on Dowex-1 (formate)

15 cm. × 0·72 cm.² in size, with a longer column (20 cm.) the resolution was little improved. The effluent was collected in 5 ml. portions (about 300 in each run) which were examined spectrophotometrically at 260 mμ and, in some instances,

radioactive material, performed in a distant laboratory with consequent difficulty in supervision, the runs were shortened by using only two solvents (4N-formic acid followed by 4N-formic acid containing M-ammonium formate), with resulting loss of resolution in the later stages (Expt. 2 of Table 1).

Some of the fractions thus obtained were rechromatographed after removal of water and formic acid by freeze-drying; if the fraction also contained ammonium formate, this was removed by the cautious use of an infrared lamp in

the concentration of adenosine deaminase. These findings are discussed with particular reference to the hypothesis that xanthine oxidase is a limiting factor in purine catabolism.

REFERENCES

ALLARD, C —(1955) *Canad Cancer Conf.*, 1, 319
BASS, A. D., TEPPERMAN, J., RICHERT, D. A AND WESTERFELD, W. W.—(1950) *Proc. Soc. exp Biol., N.Y.*, 73, 687
BEGG, R. W.—(1955) *Canad Cancer Conf.*, 1, 237
BERGEL, F, BRAY, R. C, HADDOW, A. AND LEWIN, I.—(1957) in *Chemistry and Biology of Purines* (Ciba Foundation Symposium), ed. Wolstenholme, G E. W. and O'Connor, B M. London (J and A. Churchill), p 256.
CHRISTMAN, A A —(1952) *Physiol Rev*, 32, 303
GREENSTEIN, J P, CARTER, C. E AND LUTHARDT, F M —(1946) *J nat. Cancer Inst*, 7, 47.
KALCKAR, H. M.—(1947) *J biol. Chem.*, 167, 461
KOROTKORUCHKO, V. P.—(1953) *Biokhem. Zh.*, 25, 173
DE LAMIRANDE, G AND ALLARD, C —(1957) *Proc Amer. Ass Cancer Res.*, 2, 224
LEWIN, I., BERGEL, F, BRAY, R. C, HADDOW, A AND LEWIN, I —(1957) *Ibid.*, 2, 226.
REID, E. AND O'NEAL, M. A.—(1956) *Brit. J. Cancer*, 10, 587
Idem AND LEWIN, I—(1956) *Biochem. J.*, 64, 730
Idem AND STEVENS, B. M.—(1958) *Ibid*, in press
SCHNEIDER, W. C AND HOGEBOOM, G. H.—(1952) *J biol Chem*, 195, 161.
Iidem, SHELTON, E AND STRIEBICH, M. J.—(1953) *Cancer Res.*, 13, 285
WESTWEFELD, W W, RICHERT, D. A. AND HILLINGER, M. F.—(1950) *Ibid.*, 10, 486.

final counting, this amount being in the range 2–8 μc, of orotic acid as precursor and with an interval exceeding 1 hr. between injection and autopsy. With a long interval (20 or 30 hr.), a comparison of different doses within the range 0 5–8 μc indicated that the percentage recovery in the RNA of microsomal and supernatant fractions decreased somewhat as the dose was increased. However, the decrease was not marked in relation to day-to-day variations in percentage recovery, and moreover the dosage within each group was the same for adrenalectomized rats as for the controls. Accordingly, no attempt has been made to correct for differences in dosage from one experiment to another. In experiments with an interval of only 40 min. or 1 hr., high doses of orotic acid (up to 40 μc, i.e. 6 3 mg. of orotic acid) were employed so as to ensure measurable labelling of microsomal RNA and of uridine compounds in the acid-soluble fraction; in some of these experiments the dose may have approached 'saturation level'.

Since the incorporation of orotic acid at short time intervals was low but accurately measurable, logarithmic axes have been adopted in plotting the results. These are considered in terms of percentage recovery of injected isotope except where their assessment in terms of specific activity is advantageous; the amounts and specific activities of the pyrimidines were not actually determined. It appeared appropriate in experiments requiring a standardized interval to adopt an interval of 135 min. after injection of orotic acid, as previously (Reid et al. 1956).

In experiments with leucine the standardized interval was 90 min. Data obtained at shorter time intervals (15 and 40 min.) are also given below: here L-leucine (generally labelled with ¹⁴C, Radiochemical Centre, Amersham) was employed in place of DL-[1-¹⁴C]leucine, usually at a dosage of 0 063 mg. equivalent to 6 μc.

Injection of unlabelled orotic acid. Intact rats were given massive doses of orotic acid (Genatosan Ltd., Loughborough, Leics.) to ascertain the effect upon liver RNA levels. The compound was given as an almost saturated solution in water at 37° and at pH 8 5 (3 mg./ml.), 5 ml. was injected intraperitoneally, followed by four similar injections at hourly intervals, and the rats were killed 2 hr. after the last injection. Control rats were given water containing a trace of sodium bicarbonate.

RESULTS

Liver ribonucleotides

Effects of improvements in technique. Since Hurlbert & Potter (1954) and Hurlbert et al. (1954) do not emphasize that replication of their results depends on scrupulous adherence to their techniques, as became evident during the present experiments, the data for control rats (Tables 1 and 2) may usefully be considered from this aspect before the effects of adrenalectomy are discussed. With practice in techniques it became possible to treat the tissue more expeditiously before extraction and, in particular (Expts. 4c et seq.), to grind the frozen tissue to a powder before extraction. As is evident from the data for control rats, in the earlier experiments there were post-mortem changes, namely decreases in the levels of triphosphates (ATP, UTP,

GTP) and of certain diphosphates and their derivatives [adenosine diphosphate (ADP), UDPglucose, UDPglucuronic acid and UDP] together with increases in the levels of certain monophosphates [adenosine monophosphate (AMP) and GMP] and possibly of TPN. On the other hand, the levels of DPN, of UMP and of UDPacetylglucosamine were remarkably constant throughout. In general, the patterns obtained in the final experiments quantitatively resembled those of Hurlbert et al. (1954) and of Schmitz et al. (1954).

At the outset of the study it was hoped that nucleotide levels could be determined in individual fractions isolated from sucrose homogenates. However, it soon became evident that considerable breakdown of nucleotides occurs in the course of separation of nuclei and mitochondria. This conclusion is, of course, in accord with the later findings described above.

Effects of adrenalectomy. There was a striking decrease in the amounts of uridine nucleotides 2–3 weeks after adrenalectomy (Tables 1 and 2). Rechromatography of fraction (5) of Table 1 showed no change in IMP but a highly significant decrease in UMP (P < 0 5 %; Table 2). The results for the labile compound UDPglucose (and possibly those for ADP) suggest that adrenalectomy may decrease its level *in vivo* but minimize its breakdown *post mortem*. The results for UDP, UDPglucuronic acid and UTP (Table 2) indicate marked decreases after adrenalectomy; here there is the difficulty that ribonucleoside diphosphates and their derivatives may undergo some breakdown after the initial isolation (Hurlbert et al. 1954). However, the total recovery of uridine nucleotides from fraction (11) showed a significant decrease (P < 5 %) after adrenalectomy (Table 2). In general, the decreases in the various uridine nucleotides of the liver appeared to exceed 50 % except with UDPacetylglucosamine (29 % decrease) and UDPglucose.

The results for UMP and UDPacetylglucosamine in control rats are very consistent, and the single experiment with adrenalectomized rats 3 days after operation permits the conclusion that these compounds are undiminished after only 3 days; it also appears that the amount of UDPglucose is undiminished or even elevated.

The marked decrease in the uridine nucleotide pool is not reflected in the results for adenosine and guanosine nucleotides, with the possible exception of the triphosphates. Indeed, the results for AMP, the amount of which was affected by changes in technique, showed a small but significant increase (7 %; P < 5 %) when comparison was made between fractions from adrenalectomized rats and fractions from controls studied under similar conditions. No conclusions could be drawn as to cytidine nucleotides because of analytical difficulties.

the final stage of drying as in the experiments of Hurlbert *et al.* (1954) with the aid of a mercury-diffusion pump to achieve a higher vacuum. Rechromatography of hetero-

after the rechromatography.

nucleotides (Sigma Chemical Co., St Louis, Mo., U.S.A.) and by examination of the peaks with respect to the ratio of the extinctions at 275 and 260 mμ. The chromatograms of the crude tissue extracts showed certain minor qualitative

rechromatography (even with the authentic nucleotides in place of the tissue extract), but *uridine 5'-pyrophosphate*

triphosphate (UTP), and UDP and its glucuronic acid derivative merged with adenosine triphosphate (ATP).

Diphosphopyridine nucleotide (DPN) and its reduced form (DPNH) were estimated in freshly excised liver samples by the use of alcohol dehydrogenase according to Sprites & Eichel (1954). The rats were killed by decapitation, the values tending to be lower if Nembutal (pentobarbital sodium) was used.

Isotopic studies

The number of rats used in each experiment is stated in the legends to the figures; in general each point is derived from data for two to four rats. Adrenalectomized rats were always studied simultaneously with control rats (intact or sham-operated) because of possible day-to-day variations in environment or technique.

In some experiments the interval between adrenalectomy and injection of isotope was deliberately varied. Since these experiments, which are described below, showed that the effects of adrenalectomy were quantitatively dependent on the length of this interval, it was necessary to standardize this where other variables were to be investigated. The length of the interval was 12–15 days unless otherwise stated

Isolation of fractions. As in the previous study (Reid *et al.* 1956), homogenates of the liver samples removed at autopsy were centrifuged to give nuclear and cytoplasmic fractions.

Further centrifuging of the cytoplasmic fraction gave a mitochondrial fraction (with 'fluffy layer'), which was discarded, a microsomal fraction and a supernatant fraction, i.e. the material unsedimented after 90 min at 14 500 g In some experiments the latter fraction was centrifuged for 60 min at 145 000 g to sediment an ultracentrifugal fraction.

In some instances supernatant fractions were further fractionated by procedures other than ultracentrifuging; here each point on the figures was obtained not with several

supernatant fractions, but usually with a pooled fraction from a pair of rats which had been similarly treated Ammonium sulphate precipitation was carried out by addition of saturated (NH₄)₂SO₄ solution at 0–3°, with high-speed centrifuging (10 min. at 14 500 g) so as to give a clear supernatant and a compact sediment, which was not washed. Precipitation with Ca²⁺ ions (Schneider, 1946), by addition of CaCl₂ solution to a final concentration of 0 1% CaCl₂, was carried out at room temperature; high-speed centrifuging was not necessary.

analyses for RNA phosphate.

In preparing a powder for measurement of radioactivity, the initial removal of acid-soluble and lipid constituents from tissue samples was usually performed with perchloric acid solution followed by treatment at room temperature with ethanol–ether–chloroform (2:2:1, by vol.), as in the experiments of Littlefield, Keller, Gross & Zamecnik (1955). The use of hot ethanolic solvents, as in the previous study (Reid *et al.* 1956), was less convenient and, moreover, might

soluble radioactivity (see below) or when nuclear preparations were being treated; the latter tended to become fibrous if treated with perchloric acid.

Plating of liquid samples. Liquid samples were dried and counted at slightly less than infinite thickness, with a standard amount of carrier sufficient to minimize differences due to any variations in the amount of tissue solid. Hurlbert & Potter (1954) used ammonium trichloroacetate for this purpose; but with this alone, erratic results due to uneven drying have now been encountered, even with nickel planchets in place of polyethylene ones. With BaSO₄ as an additional constituent, as described below, duplicates seldom differed by more than 5%.

With cytoplasmic fractions, the tissue extracts, containing 7% (w/v) trichloroacetic acid, were prepared for counting by pipetting 0 1 ml into the well of a polyethylene planchet (2 cm²), followed by 0 13 ml. of 0 5n-Ba(OH)₂ solution and 0 013 ml. of 2n-H₂SO₄ solution Finally an excess of aq NH₃ soln. (sp.gr. 0 88) was added, with stirring so as to disperse the precipitate of BaSO₄. The contents of the planchet were carefully taken to dryness by exposure for about 3 hr. to an infrared lamp.

With fractions obtained by anion-exchange chromato-

added to bring the total amount of formic acid present to 1·15 m-moles (e.g. 0 05 ml. added if none already present; 0 04 ml was added to effluents containing UMP). After addition of 0 05 ml of 0 5n-Ba(OH)₂ solution and 0 02 ml. of 2n-H₂SO₄ solution, excess of aq NH₃ soln. was added with

Injection of isotope Injections were given intraperitoneally unless otherwise stated. In general, the dosage chosen was the lowest amount compatible with accuracy in the

Diphosphopyridine and reduced diphosphopyridine nucleotides. Separate analyses for these compounds were performed with five pairs of rats, each pair consisting of an intact rat and a rat which had been adrenalectomized 2–3 weeks previously. The mean values for DPN and DPNH in the controls were 1·06 and 0·46 mg./100 g. body wt. respectively (1 mg. of DPN is equivalent to 27·7 'units' as defined in Table 1). The mean changes (with standard error) after adrenalectomy were +0·04 ± 0·142 and −0·05 ± 0·072 respectively; these changes are insignificant.

Incorporation of orotic acid in relation to time after injection of isotope

In presenting their data for the time course of labelling of the various RNA fractions, Hurlbert & Potter (1954) remark, 'The data do not offer an accurate kinetic record because too few animals were used to offset the large variations in utilization of the dose in these experiments. The reason for these variations has not been determined...,' The more extensive data now obtained show not only quite consistent changes with time in spite of day-to-day variations, but also good agreement with the data given by Hurlbert & Potter (1952, 1954) for the labelling of microsomal, supernatant, nuclear and acid-soluble fractions analogous to those now prepared.

Microsomal and supernatant fractions. In control rats (Fig. 1), maximal labelling occurred at about 30 hr. with the supernatant fraction, and at about 40 hr. with the microsomal fraction, the labelling of the latter being low in the first few hours after the injection. The labelling of both fractions remained high for at least the next 3 days.

If the RNA of the supernatant fraction were the precursor of microsomal RNA, the descending portion of the specific-activity curve for the former should intersect that for the latter at its maximum (Zilversmit, Entenman & Fishler, 1943). The data for control rats in Fig. 1 are compatible with this possibility, the curves for percentage recovery being also a rough measure of specific activity since microsomal and supernatant fractions as now prepared contain similar amounts of RNA (about 8 mg./100 g. body wt.; cf. Reid, 1956; Reid et al. 1956)[*] Consideration is given below to the apparent plateau in the ascending portion of the curve for the supernatant fraction.

* In Table 3 of Part 3 (Stevens & Reid, 1956), the values refer, not to RNA, as incorrectly stated, but to RNAP.

Table 2 *Rechromatography of heterogeneous ribonucleotide fractions*

See Table 1 for abbreviations and for method of expressing results. Fraction nos. are those indicated in Table 1

Fraction no.	Expt. no.	UMP	IMP	UDP	UDP-glucuronic acid, CTP	UTP	GDP	CTP
					Control rats			
	3c	9·0	5·4	—	—	—	—	—
	4c	12·9	8·5	—	—	—	—	—
	5c	9·5	4·5	—	—	—	—	—
				Adrenalectomized rats (2–3 weeks after operation)				
(5)	3a	5·2	4·5	—	—	—	—	—
	4a	4·6	6·5	—	—	—	—	—
	4a'	4·7	4·7	—	—	—	—	—
	6a	4·2	8·2	—	—	—	—	—
				Adrenalectomized rats (3 days after operation)				
	5a'	12·2	—	—	—	—	—	—
					Control rats			
	3c*	1·3	—	0·2	6·0 *	—	—	—
	4c	0·9	—	2·0	11·7	—	—	—
	5c	9·4	—	2·5	17·5	—	—	—
(11)				Adrenalectomized rats (2–3 weeks after operation)				
	4a'	0	—	0·0	3·8	—	—	—
	5a*	1·8	—	0·6	2·0	—	—	—
					Control rats			
	5c	0	—	0·3	—	3·5	1·5	0·9
(13)				Adrenalectomized rats (2–3 weeks after operation)				
	5a	0	—	0·3	—	0·9	0·8	—
	5a*	0	—	0·4	—	0·9	<0·2	0·5

* This experiment, in which the conditions were not optimum, has been disregarded in assessing the effect of adrenalectomy on UDP and UDPglucuronic acid.

Table 1. *Chromatography of ribonucleotides in perchloric acid extracts of rat liver*

Control and adrenalectomized rats were studied alternately as indicated by the Expt. nos. In series 1 the rats were killed with Nembutal instead of by decapitation. In series 1–3 the liver was macerated while in a semi-thawed state before extraction, whereas in series 4 and 5 the liver was ground to a powder while frozen (liquid nitrogen), the time between decapitation and freezing of the liver being particularly short (1 min.) in series 5. In view of these improvements in technique, the effects of which are discussed in the text, each run with adrenalectomized rat is, in general, comparable only with the corresponding control run. All quantities refer to an amount of liver corresponding to 100 g. body wt. and are expressed in arbitrary units, namely the calculated optical density (at 260 mμ with water as blank and 1 cm. light path) for the material in the fraction if present in only 1 ml. volume. (Similar units are conventionally used to express the molar extinction coefficients of pure compounds.) Abbreviations used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; MP, monophosphate; DP, diphosphate; TP, triphosphate; A, adenosine; G, guanosine; I, inosine; C, cytidine; U, uridine (Thus UDP denotes uridine 5'-pyrophosphate). These abbreviations, and those for unidentified compounds ('C.3', 'J.2', etc.), are identical with those of Hurlbert et al. (1954). 'post-ADP' is a peak immediately following ADP, which was not labelled by these authors. For each fraction, minor constituents are italicized and trace constituents are ignored.

Fraction no.	(1) DPN, CMP	(2) AMP	(3) TPN	(4) GMP	(5) UMP, 'C.3', IMP, CDP, 'C.4'	(6) ADP	(7) 'Post-ADP'	(8) UDP-acetylglu-cosamine	(9) UDP-glucose	(10) GDP, 'G.2', 'G.3', 'G.4'	(11)* UDP-glucurone acid, UDP, GTP	(12)* ATP, 'J.3', 'J.2'	(13) UTP, GTP, 'K.1'
Expt. no.						Control rats							
1c	24.4	62.7	6.5	12.0	31.8	22.0	9.6	10.2	7.8	18.4	21.8	—	7.9
2c	23.9	66.8	4.5	11.7	32.3	25.4	—	{14.1}	{14.1}	15.7	16.1	—	—
3c	28.0	73.9	6.4	13.2	35.0	34.5	8.3	11.6	12.9	17.7	8.7	12.2	3.7
4c	23.0	49.5	4.8	8.1	36.8	60.4	9.7	13.5	19.8	22.2	23.6	22.9	14.6
5c	22.5	43.0	3.2	5.0	20.6	51.1	6.0	11.0	ca. 17	—	35.8	23.7	12.2
						Adrenalectomized rats (2–3 weeks after operation)							
1a	—	66.6	7.1	11.1	25.4	34.9	14.1	9.3	13.5	19.9	8.1	11.8	7.3
2a	27.8	68.0	8.1	10.6	40.5	30.4	—	{20.7}	{20.7}	11.5	31.0	—	—
3a	33.4	76.8	7.7	13.3	27.4	36.0	10.5	8.9	14.8	—	—	—	—
4a	21.4	57.0	2.8	8.1	22.4	{42.1}	{4.9}	{7.0}	{12.5}	19.6	15.3	10.5	10.3
4a'	25.0	56.8	3.0	7.4	19.5								
5a	25.2	45.4	4.2	6.7	25.2	48.8	7.6	8.0	14.7	23.2	36.8	14.1	9.9
5a'	—	—	—	—	—	—	{7.7}	7.7	12.7	—	17.5	—	8.1
						Adrenalectomized rats (3 days after operation)							
5a'	—	—	—	—	28.4	—	—	13.6	34.0	—	—	—	—

* These fractions were never well separated, and the values given in some instances for the two fractions separately may not be individually accurate.

respect to the amount of RNA (Table 3), this being increased by 16 %, whereas the average increases for the first precipitate and for the final supernatant were 10 and 2·5 % respectively.

Other experiments (not illustrated) were performed with three concentrations of $(NH_4)_2SO_4$, namely 33, 38 and 55 % saturation. The activity values showed the same trends as in the above experiments, the second precipitate being intermediate in behaviour between the first and third precipitates.

Barnum & Huseby (1950) mention preliminary experiments in which the RNA of the supernatant fraction, from mice given ^{32}P, was fractionated by $CaCl_2$ (0·4 %) or by isoelectric precipitation: 'It was invariably possible to obtain a small non-precipitable fraction with a comparatively high
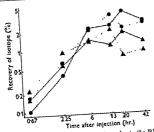
Fig. 4. Incorporation of injected orotic acid into the RNA of subfractions separated from supernatant fraction, namely precipitate (●) and supernatant (▲) obtained by addition of $CaCl_2$ to 0·1 % (w/v), in control rats (——) and adrenalectomized rats (·····). Each point at 13 hr. represents two experiments, and the other points each represent one experiment, total no. of rats used in preparation of supernatant fraction 'pools', 25.

relative specific activity....The reproducibility of these fractions was poor, but it is possible to conclude that the supernatant PNA [i.e. RNA] contains a small fraction whose relative specific activity initially increases much more rapidly than does that of the microsomal PNA.' Accordingly, $CaCl_2$ has now been employed, with the results shown in Fig 4. Since little more than one-half of the original RNA was precipitated (Table 3), presumably less than in the experiments mentioned by Barnum & Huseby, the curves representing percentage recovery are also a rough measure of specific activity. The data for both adrenalectomized and control rats do in fact show a difference between the precipitable RNA and the non-precipitable RNA, the specific activity of the latter being about double that of the former at the shortest time interval (40 min.) and half that of the former at long time intervals (30 and 42 hr.). Since, however, the peak of labelling occurred at the same time for both subfractions, as for the precipitates obtained with $(NH_4)_2SO_4$, it cannot be concluded that one is the direct precursor of the other.

The data of Table 3 show that an increase in the amount of RNA after adrenalectomy occurs more consistently with the precipitable subfraction than with the non-precipitable subfraction.

The specific activity of the non-precipitable RNA at 40 min. after the injection was little altered by the use of lower final concentrations of $CaCl_2$ (0·06 or 0·075 % in place of 0·1 %). Concentrations higher than 0·1 % did not increase the amount of RNA precipitated.

Isoelectric precipitation was performed in one experiment, with a short interval after isotope injection, but the specific activity of the non-precipitable RNA was no higher than that of the precipitable RNA, in contrast with the results mentioned by Barnum & Huseby (1950). The trial of alumina gel ($C_γ$) as an alternative means of fractionation was abandoned when similar values were found, at long or short intervals after isotope in-

Table 3 *Ribonucleic acid in subfractions separated from supernatant fraction of rat liver*

Values are expressed as mg. of RNA/100 g. body wt. The last column gives the mean differences (adrenalectomized rats minus controls) ± S.E. of differences (with no. of degrees of freedom in parentheses)

	Control rats: mean value	Adrenalectomized rats: mean difference from controls
$(NH_4)_2SO_4$ fractionation		
1st precipitate (35 % of saturation)	2·7	+0·27 ± 0·135 (6)
2nd precipitate (35 % to 55 % of saturation)	4·9	+0·79 ± 0·291 (7)*
Supernatant	1·9	+0·05 ± 0·123 (6)
$CaCl_2$ fractionation		
Precipitate (0·1 % $CaCl_2$)	6·0	+0·82 ± 0·339 (8)†
Supernatant	4·0	+0·53 ± 0·459 (8)

* $P < 5$ %   † $P < 10$ %.

The labelling of the supernatant fraction was higher with adrenalectomized rats than with control rats at all times up to 13 hr., and lower at all subsequent times. However, the labelling of the microsomal fraction was lower in adrenalectomized rats at all times, and especially after 13 hr. or longer. Even if allowance is made for the previous finding (Reid, 1956; Stevens & Reid, 1956) that supernatant fractions from adrenalectomized rats contain somewhat more RNA than those from controls, the curves for adrenalectomized rats are also compatible with the hypothesis that the RNA of the supernatant fraction is the precursor of microsomal RNA.

One assumption underlying this postulate is that the microsomal and supernatant fractions are each homogeneous with respect to RNA. It appears from the following experiments that the supernatant fraction is not entirely homogeneous in this respect.

Subfractions obtained from supernatant fraction The results for subfractions obtained by ultracentrifuging are shown in Fig 2. It is evident that there is no striking difference in the time course of labelling between the sedimented ('pellet') RNA (this being about one-half of the original RNA) and the non-sedimented ('supernatant') RNA, and that the subfractions are equally affected by adrenalectomy except, perhaps, with respect to the labelling at 20 hr.

In the next experiment (Fig. 3), $(NH_4)_2SO_4$ was added to 35 % of saturation, and then to 55 % of saturation. The results were somewhat variable, but
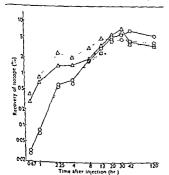
it does appear that at short time intervals, in contrast with long time intervals, the precipitate obtained at 35 % of saturation becomes labelled as rapidly as the other subfractions and has a higher specific activity (cf. RNA values in Table 3). There is no indication that adrenalectomy affects one of the fractions selectively with respect to labelling. However, the precipitate obtained between 35 and 55 % of saturation may be particularly affected with
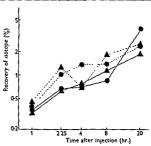
Fig 2. Incorporation of injected orotic acid into the RNA of subfractions separated from supernatant fraction, namely ultracentrifugal pellet (●) and ultracentrifugal supernatant (▲), in control rats (——) and adrenalectomized rats (- - - -). Total no. of rats, 22.
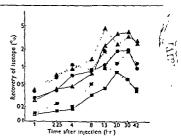
Fig. 3. Incorporation of injected orotic acid into the RNA of subfractions separated from supernatant fraction, namely precipitate obtained by ammonium sulphate at 35% of saturation (●), precipitate obtained from 35 to 55% of saturation (▲), and supernatant (■), in control rats (——) and adrenalectomized rats (- - - -). Each point represents one experiment; total no of rats used in preparation of supernatant fraction 'pools', 28.

Incorporation of leucine in relation to time after injection of isotope

Whole cytoplasmic fraction. It was conceivable that a delayed absorption of injected DL-leucine after adrenalectomy was responsible for the decrease previously found (Reid *et al.* 1956) in the labelling of cytoplasmic protein. Since each of the cytoplasmic elements shared in this decrease, only the whole cytoplasmic fraction has now been reinvestigated. In the previous experiments, with an interval of 90 min. between injection and autopsy, the recovery of isotope (corrected for the presence of the 'inert' D isomer) in cytoplasmic protein was 9% in rats adrenalectomized 2 weeks previously, as compared with 10·2% in corresponding controls. With L-leucine, mean values of 7·1 and 7·25% respectively have now been found 40 min. after injection, and values of 9·2 and 10·7% have been found 15 min. after injection, the latter high values being perhaps a reflexion of day-to-day variation. It was anticipated that liver excised 15 min. after the injection would contain an appreciable amount of radioactivity in the form of free leucine; but the amount thus recovered was only about 0·1%, this amount being unaffected by adrenalectomy. It is, however, evident that the slight decrease in protein labelling after adrenalectomy is manifest even at relatively short time intervals.

Subfractions from microsomal fraction. Littlefield *et al.* (1955) have claimed that in the first few minutes after intravenous injection of a labelled amino acid, the activity of the microsomal protein which remains insoluble after deoxycholate treatment and which is rich in RNA is markedly higher than that of the solubilized protein. An attempt has now been made to ascertain whether adrenalectomy affects this initial labelling of the insoluble protein.

In preliminary experiments it was confirmed that a pellet consistently rich in RNA could be obtained by deoxycholate treatment of microsomal fractions, provided that the sedimented fractions were dispersed in a small volume of water before addition of the deoxycholate solution rather than dispersed directly in the latter. In the next experiment, liver was exised from control and adrenalectomized rats 3 min. after intravenous injection of labelled leucine, and microsomal fractions were rapidly isolated and treated with deoxycholate as in the experiments of Littlefield *et al.* (1955). In agreement with these authors, the specific activity of each pellet was higher than that of the corresponding supernatant (Table 5). However, the individual rats varied so widely that it was impossible to assess whether adrenalectomy had any effect on the labelling. There was no consistent effect of adrenalectomy on the yield or RNA content of the pellet or supernatant.

Incorporation of orotic acid and of leucine in relation to time after adrenalectomy

In the following experiments, adrenalectomized rats were studied at different times after operation; in isotopic experiments the time after injection of isotope was standardized as stated in the Experimental section.

Amount and labelling of ribonucleic acid. It is evident from Fig. 6 that the effect of adrenalectomy in increasing the amount of RNA in the supernatant fraction is not manifest 1 day after operation, but is fully manifest at 3 days. The increase in the incorporation of orotic acid into the RNA of the supernatant fraction is already manifest at 3 days, but continues to increase with time. On the other hand,

Table 5. *Labelling of microsomal subfractions 3 min. after intravenous injection of leucine*

... protein (the counting being at infinite thickness)

		Deoxycholate pellet	Deoxycholate supernatant
Control rats	Intact	2·4	2·2
	Intact	0·55	0·12
	Sham-operated	0·53	0·17
Adrenalectomized rats		2·4	0·34
		2·2	0·66
		0·57	0·13
		2·3	1·4
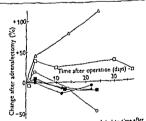
Fig 6. *Changes after adrenalectomy, related to time after operation:* □, amount of RNA (/100 g body wt.) in the supernatant fraction; △, incorporation of orotic acid into the RNA of the supernatant fraction; ○, incorporation of orotic acid into the RNA of the microsomal fraction; ■, amount of protein (/100 g body wt.) in the microsomal fraction; ●, incorporation of leucine into protein of microsomal fraction; ▲, incorporation of leucine into protein of whole cytoplasmic fraction. The total nos. of rats were respectively 55, 22, 22, 30, 24 and 23.

jection, for the ratio of the specific activity of the non-adsorbed RNA to that of the RNA eluted from the gel by 0·15M-Na₂HPO₄ solution.

Nuclear fraction. Data for nuclear fractions, containing any cells left unbroken after the two treatments in the homogenizer, are given in Fig. 5. In view of the heterogeneity of the RNA in this crude fraction, no conclusions can be drawn from the recovery values, or even from specific activity values, concerning the possible role of nuclear RNA as the precursor of cytoplasmic RNA. It can, however, be concluded that the increase in the labelling of supernatant-fraction RNA after adrenalectomy is not attributable to more rapid entry of label into nuclear RNA with consequent faster transfer to the supernatant fraction.

In a few experiments, crude nuclear fractions were treated repeatedly with citric acid solution as in the experiments of McIndoe & Davidson (1952). With these partially purified nuclei, as with the crude fractions, there was no indication of an increase in activity after adrenalectomy at the times studied (1, 4 and 20 hr. after injection).

Acid-soluble fraction. The recovery of activity in this fraction, as shown in Fig. 5, was not consistently higher after adrenalectomy. Evidently adrenalectomy does not increase the proportion of injected orotic acid which enters the liver. It was of interest to know whether the pattern of labelling of the various constituents of the acid-soluble fraction is altered by adrenalectomy. Anion-exchange chromatography was therefore carried out with tissue excised 40 min. after injection of orotic acid in high dosage (Table 4). At this time the labelling of cytidine derivatives, which are present in relatively small amounts (Hurlbert & Potter, 1954), is low in comparison with that of uridine derivatives (Hecht & Potter, 1956). From the few experiments performed, it appears that the rate of conversion of orotic acid to uridine derivatives is unchanged or possibly somewhat decreased after adrenalectomy. Orotic acid given in low dosage is not detectable in the liver even 30 min. after injection, its conversion into uridine compounds being very rapid (Hurlbert & Potter, 1952, 1954). Its identification in the liver extracts now studied was based on its position

(judged by the radioactivity and compared with that of authentic material) on the anion-exchange chromatogram and on a subsequent paper chromatogram with the propan-2-ol–water–HCl solvent of Wyatt (1951). In the former system orotic acid was not separable from ADP.

Labelling of liver deoxyribonucleic acid and of kidney ribonucleic acid. With the short time interval (135 min.) employed in the previous study (Reid *et al.* 1956), the labelling of liver deoxyribonucleic acid (DNA) was very low, and that of kidney RNA was too variable for any effect of adrenalectomy to be manifest. These aspects have now been reinvestigated, with the same dose (8 μc) but with a longer time interval (20 hr.). It was again found that the labelling of DNA was barely measurable, as also found by Hurlbert & Potter (1954), and that the labelling of the RNA in the whole kidney or in the supernatant fraction was very variable.
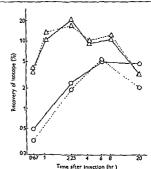
Fig. 5. Incorporation of injected orotic acid into the RNA of the nuclear fraction (O), and recovery of radioactivity in the acid-soluble fraction (△), in control rats (——) and adrenalectomized rats (- - - -). Total no. of rats, 16 (nuclear fraction) or 30 (acid-soluble fraction).

Table 4. *Radioactivity of constituents of acid-soluble fraction of rat liver after large doses of orotic acid*

Rats were killed 40 min. after injection of (6-¹⁴C)orotic acid. Techniques used for chromatography and for measuring radioactivity are described in the text; techniques in Expts. C and A differed in minor respects from those in C′ and A′. Values for the constituents (see Table 1 for abbreviations) represent percentage recovery of injected isotope.

	Expt.	UMP	Orotic acid	UDPacetyl-glucosamine, UDPglucose	UDP, UDPglucuronic acid
Control rats	C	2·4	0·4	2·6	—
	C′	1·45	1·5	1·6	0·8
Adrenalectomized rats	A	1·25	2·4	2·1	0·3
	A′	1·45	1·75	1·5	—

earlier experiments that liver contains a 'pool' of uridine nucleotides which rapidly become uniformly labelled after injection of orotic acid (Hurlbert & Potter, 1954; Schmitz, Hurlbert & Potter, 1954). The view that orotic acid, despite its apparent absence from normal liver (Hurlbert & Potter, 1952, 1954), is a physiological precursor of uridine nucleotides is supported by evidence from other laboratories (Lieberman, Kornberg & Simms, 1955; Hurlbert & Reichard, 1955; Wu & Wilson, 1956). Orotic acid is also a precursor of cytidine nucleotides, although the study of this conversion is handicapped by the difficulty of analysing the relatively small amounts of these nucleotides in liver (Hurlbert & Potter, 1954).

It has now been shown that at least some of the uridine nucleotides are markedly decreased in amount 2–3 weeks after adrenalectomy. That this observation does not hold for adenosine or guanosine nucleotides is suggested by the data for AMP and GMP, the amount of the former being actually greater after adrenalectomy. The changes in uridine nucleotide levels may have wide implications with respect to processes such as glucuronide synthesis. Here it need only be pointed out that if uridine nucleotides are in fact immediate precursors of supernatant-fraction RNA, as is discussed below, an increased incorporation of injected orotic acid into this RNA does not signify an increased rate of synthesis of RNA unless the increase in incorporation outweighs the decrease in the uridine nucleotide pool. Consideration of Table 2, together with Fig. 6, suggests that the rate of synthesis of supernatant-fraction RNA 2–3 weeks after adrenalectomy is increased not by about 100%, as Fig. 6 would suggest, but by less than 30%. That some increase does in fact occur is indicated by the data obtained 3 days after adrenalectomy, as is discussed below. (If supernatant-fraction RNA is formed, not from nucleotides but from nuclear RNA, the increased labelling of supernatant-fraction RNA may truly reflect increased synthesis, since adrenalectomy tends to increase, rather than diminish, the amount of RNA in the nuclear fraction [Reid, 1956].)

Obviously there may be other variables which could affect the interpretation of incorporation values in terms of rates of RNA synthesis. Adrenalectomy might conceivably have altered the rate or extent of the conversion of injected orotic acid into uridine nucleotides in liver. However, the possibility that adrenalectomy increases the amount of isotope entering the liver is ruled out by the results for the acid-soluble fraction (Fig. 5), the radioactivity of which is largely attributable to uridine nucleotides (Hurlbert & Potter, 1954). The results for radioactive uridine nucleotides in this fraction (Table 4), 40 min. after injection of orotic acid in a high dosage such that free orotic acid could be detected in the

liver, suggest that the formation of uridine nucleotides is slowed rather than accelerated by adrenalectomy. Evidently, increased recovery of labelled material in supernatant-fraction RNA after adrenalectomy is not a consequence of a rise in the number of radioactive molecules in the acid-soluble fraction

Since the amount of RNA in the supernatant fraction has already risen to a maximum 3 days after adrenalectomy (Fig. 6), an increased rate of synthesis of this RNA 2–3 weeks after adrenalectomy must be balanced by an increased rate of catabolism. This conclusion is compatible with the data of Fig. 1 and with data for ribonuclease levels (Reid & Stevens, 1956; Stevens & Reid, 1956) which will be discussed in a later paper.

It does appear that the increase in the amount of RNA occurring as an initial effect of adrenalectomy is attributable to increased synthesis rather than to decreased catabolism (Fig. 6 and Table 6), the uridine nucleotide pool being then elevated (with no increase in its labelling). This increased synthesis might be due to an increase in the amount of the enzyme(s) concerned in the formation of RNA from ribonucleotides. Daoust & Cantero (1956) suggest that 'the rate-limiting step in the synthesis of nucleic acids is not the formation of free nucleotides but rather the building up of nucleic acids from individual nucleotides'. The data of Table 7 are not incompatible with this view.

As Hurlbert & Potter (1954) have pointed out, the time course of labelling of the different fractions (cf. Figs. 1, 5) is compatible with the view that nuclear RNA is formed from acid-soluble ribonucleotides, but does not suggest a direct relationship between the latter and cytoplasmic RNA as has been assumed in the above discussion. The suggestion that nuclear RNA is the direct precursor of supernatant-fraction RNA (Jeener & Szafarz, 1950) is not supported by observations from other laboratories (Barnum, Huseby & Vermund, 1953; Smellie, McIndoe, Logan, Davidson & Dawson, 1953; Sacks & Samarth, 1956; Brachet, 1956), although Smellie et al. recognize the possibility that nuclear RNA contains several constituents, one of which may be such a precursor. It is, however, possible that the supernatant fraction contains at least two types of RNA, one of which is in fact ...

the supernatant fraction, in accordance with ... preliminary experiments mentioned by Barnum & ...

... sub-
... their
... cleus
in vitro. Studies in which RNA was isolated at a fixed time after isotope injection and was deliber-

the incorporation into the microsomal fraction is undiminished after 3 days, but subsequently decreases.

Amount and labelling of protein. Re-examination of the data of Reid *et al.* (1956) showed that adrenalectomy had consistently decreased the amount of microsomal protein as judged by the yield of the residue remaining after removal of acid-soluble material and lipids. As is now shown (Fig. 6), both this decrease and the decrease in the incorporation of leucine into the protein of the microsomes or the whole cytoplasm become manifest gradually, no decrease occurring only 3 days after operation.

Catabolism of ribonucleic acid

The possible effect of adrenalectomy on the catabolism of RNA cannot readily be assessed from the data of Fig. 1, the amount of RNA and its apparent rate of synthesis being themselves affected by adrenalectomy. The following approach was adopted. Adrenalectomy was performed 8 hr. after injection of isotope, presumably without effect on the time or extent of maximal labelling since, as was shown in Fig. 6, the amount of RNA in the supernatant fraction is not increased 1 day after operation. During the following 2 days the amount of this RNA rises to a maximum. If this rise were the consequence of a decrease in the catabolism of RNA, supernatant fractions prepared from the adrenalectomized rats after a few days' interval should have retained their labelling more fully than those from controls.

Table 6. *Catabolism of ribonucleic acid shortly after adrenalectomy*

Experimental rats were adrenalectomized, and the control rats sham-operated, 8 hr. after injection of 2·3 μc of orotic acid; the rats were killed after 5 days. Values represent percentage recovery of injected isotope

	Microsomal fraction	Supernatant fraction
Control rats	6·9	3·75
	4·2	3·45
	5·0	3·2
Adrenalectomized rats	4·95	2·7
	4·1	2·35
	4·9	3·95

As is shown in Table 6, the labelling of the supernatant fraction after 5 days was no greater for the adrenalectomized rats than for the controls. The labelling of the microsomal fraction was also little affected, as would be expected in view of the observations that adrenalectomy does not affect either the amount of microsomal RNA (Reid, 1955), or its labelling shortly after operation (Fig. 6).

Effect of massive doses of orotic acid on liver ribonucleic acid levels

It is possible that orotic acid, or a uridine compound derived therefrom, is a limiting factor in RNA synthesis. To test this possibility, analyses for RNA were performed on livers from intact rats given massive doses of unlabelled orotic acid. Table 7 shows that this treatment appeared to increase markedly the amount of acid-soluble RNA precursors, as judged by the ultraviolet-light extinction at 260 mμ. The amount of RNA in the supernatant fraction was slightly increased, but that in the liver as a whole tended to decrease. Preliminary chromatographic studies with the acid-soluble fractions indicated that the increase in extinction was largely attributable to an increased amount of non-acidic material, possibly nucleosides.

A possible interpretation of these data is that the increase in supernatant-fraction RNA occurred only by virtue of enhanced breakdown or depressed formation of RNA elsewhere in the cell. Although the amount of RNA in the supernatant fraction did increase in response to administration of a uridine precursor, it is nevertheless possible that the incorporation of uridine nucleotides into RNA in normal rats is to some extent limited by the amount of the enzyme or enzymes concerned.

DISCUSSION

It is probable, although not proven for liver, that nucleic acids are formed at least in part from nucleoside 5'-diphosphates (pyrophosphates) or 5'-triphosphates (Grunberg-Manago, Oritz & Ochoa, 1955; Kornberg, Lehman, Bessman & Simms, 1956). Potter, Hecht & Herbert (1956) have achieved synthesis of liver RNA *in vitro* with [14C]-labelled orotic acid or UMP as precursor, having shown in

Table 7. *Effect of massive doses of orotic acid on liver-ribonucleic acid levels in intact rats*

The last column gives the mean differences (treated rats minus controls)±s. x. of differences (with no. of degrees of freedom in parentheses).

	Control rats: mean value	Rats treated with orotic acid: mean difference from controls
Extinction at 260 mμ of acid-soluble fraction, diluted so that 1·1 is equivalent to 100 g. body wt.	0·264	+0·099±0·0106 (18)*
RNA, mg./100 g. body wt. { Whole liver	34·0	−3·1±2·52 (17)
{ Supernatant fraction	7·0	+1·0±0·46 (18)†

* *P* < 0·1%.   † *P* < 5%.

and possibly in uridine diphosphate glucose, but not in liver nucleotides in general.

2. Microsomal and supernatant fractions have been isolated from liver at different times after injection of labelled orotic acid. The results confirm that the labelling of the ribonucleic acid of the microsomal fraction is decreased after adrenal-ectomy and that of the supernatant fraction increased, and are compatible with the possibility that ribonucleic acid in the supernatant fraction is a precursor of microsomal ribonucleic acid.

3. Supernatant fractions containing labelled ribonucleic acid have been fractionated by several methods, two of which have indicated heterogeneity in this ribonucleic acid with respect to the time course of labelling. However, the labelling of the different subfractions was equally affected by adrenalectomy.

4. The labelling of nuclear and acid-soluble fractions, and of uridine nucleotides separated from the latter, shows no increase after adrenalectomy.

5. The labelling of microsomal ribonucleic acid, the labelling of cytoplasmic or microsomal protein with leucine as precursor, and the amount of microsomal protein are undiminished 3 days after adrenalectomy but decrease thereafter. The increase in the labelling of supernatant-fraction ribonucleic acid is already apparent 3 days after adrenalectomy, together with a rise in the amount of this ribonucleic acid which evidently occurs because of an increase in synthesis rather than a decrease in catabolism.

REFERENCES

Awapara, J. & Kit, S. (1954). *Endocrinology*, 55, 186
Awapara, J., Skellenger, W & Manz, N. (1955). *Tex. Rep. Biol. Med.* 13, 1.
Barnum, C. P. & Huseby, R. A. (1950). *Arch. Biochem* 29, 7.
Barnum, C. P., Huseby, R. A. & Vermund, H. (1953) *Cancer Res.* 13, 890.
Brachet, J. (1956). In *Ionizing Radiations and Cell Meta-bolism, Ciba Foundation Symp.* p. 3. London. J. and A. Churchill.
Daoust, R. & Cantero, A. (1955). *Cancer Res.* 15, 734.
Grunberg-Manago, M., Oritz, P. J. & Ochoa, S (1955) *Science*, 122, 907.
Hecht, L. I. & Potter, V. R (1956). *Cancer Res* 16, 999.
Hurlbert, R. B. & Potter, V. R. (1952). *J. biol. Chem.* 195, 257.
Hurlbert, R. B. & Potter, V. R. (1954). *J. biol. Chem.* 209, 1.
Hurlbert, R. B. & Reichard, P. (1955) *Acta chem. scand.* 9, 251.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* 209, 23.
Jardetzky, C. D. & Barnum, C. P. (1956). *Fed. Proc.* 15, 282.
Jeener, R. & Szafarz, D (1950). *Arch. Biochem.* 26, 54.
Kornberg, A., Lehman, I. R., Bessman, M. J. & Simms, E. S. (1956). *Biochim. biophys. Acta*, 21, 197.
Korner, A. & Debro, J. R. (1956) *Nature, Lond*, 176, 1067.
Lieberman, I., Kornberg, A. & Simms, E. S. (1955). *J. biol. Chem.* 215, 429.
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955) *J. biol. Chem.* 217, 111.
Logan, R. & Smellie, R. M. S (1956). *Biochim. biophys. Acta*, 21, 92.
McIndoe, W. M. & Davidson, J. N. (1952). *Brit. J. Cancer*, 6, 200.
Moldave, K. & Heidelberger, C. (1954). *J. Amer. chem. Soc.* 76, 679.
Potter, V. R., Hecht, L. I. & Herbert, E. (1956). *Biochim. biophys. Acta*, 20, 439.
Reid, E. (1955). *Nature, Lond.*, 175, 461.
Reid, E. (1956). *J. Endocrin.* 13, 319.
Reid, ... & Burnop, V. C. E. ... *ocrin.* 14, xli. ... *biol. Chem.* 223, 423.
Schmitz, H., Hurlbert, R. B. & Potter, V. R. (1954) *J. biol. Chem.* 209, 41.
Schmitz, H., Potter, V. R., Hurlbert, R. B. & White, D M. (1954). *Cancer Res.* 14, 66.
Schneider, W. C. (1946). *J. biol. Chem.* 166, 595
Smellie, R. M. S, McIndoe, W. M., Logan, R., Davidson, J. N. & Dawson, I. M. (1953). *Biochem. J.* 54, 280
Spirtes, M. A. & Eichel, H. J. (1954). *Arch Biochem. Biophys.* 53, 308.
Stevens, B. M. & Reid, E. (1956). *Biochem. J.* 64, 735
Wu, R. & Wilson, D. W. (1956). *J. biol. Chem.* 223, 195.
Wyatt, G R. (1951). *Biochem. J.* 48, 584.
Zilversmit, D. B., Entenman, C. & Fishler, M. C (1943) *J. gen. Physiol.* 26, 325.

ately degraded, the products differing in specific activity values, have been reported by Sacks & Samarth (1956), whose interpretation of the results is, however, open to question, and by Moldave & Heidelberger (1954), who found 'intramolecular heterogeneity' with respect to the labelling of RNA phosphate (and guanine?) but not, in the supernatant fraction, with respect to that of adenine and pyrimidines after injection of labelled glycine, and orotic acid.

The differences in labelling between the subfractions in the present experiments, together with the 'plateau' in the ascending portion of the curve for the whole supernatant fraction (Fig. 1), suggest the presence of two types of RNA, one of which may be the precursor of the other, the labelling of both types being enhanced by adrenalectomy. More rigorous fractionation will be required if the criteria of Zilversmit et al. (1943) for a precursor-product relationship are to be met. With orotic acid there is the inherent difficulty that RNA cytosine, as well as uracil, becomes labelled at long time intervals after isotope injection (Hurlbert & Potter, 1954); in the nuclear fraction from regenerating liver the RNA pyrimidines have become equally labelled at 12–24 hr. after isotope injection (Hecht & Potter, 1956).

Despite the latter complication and the probable heterogeneity of the RNA in each of the cellular elements, some evidence has been obtained in support of the postulate that microsomal RNA is formed from supernatant-fraction RNA (Jeener & Szafarz, 1950, Barnum et al. 1953). Whereas the specific activity values for microsomal and supernatant fractions after injection of ^{32}P showed no maxima in the experiments of Barnum et al. (1953) and similar maxima in the experiments of Smellie et al. (1953), the microsomal fractions now studied have shown a maximum, albeit broad and late, which is intersected by the descending portion of the curve for the supernatant fraction (As has been pointed out above, the curves of Fig 1 can be regarded as representing specific activity as well as percentage recovery.) Moreover, in the experiment of Table 7 the preferential increase in supernatant-fraction RNA suggests that the synthesis of this RNA precedes that of RNA elsewhere in the cytoplasm.

Closer consideration of the isotopic data from the kinetic aspect must await study of each fraction with respect to the amounts and specific activities of RNA cytosine and uracil. It may, however, be pointed out that at short time intervals after isotope injection the specific activity of the RNA in the supernatant fraction was somewhat increased after adrenalectomy, as in the previous experiments (Reid et al. 1956). The supposed translocation of RNA from the supernatant fraction to the micro-

somal fraction may therefore be retarded more than is suggested by the reduction in percentage of isotope recovered in the microsomal fraction.

The reduction in microsomal labelling, unlike the increase in the labelling of the supernatant fraction, is a late effect of adrenalectomy. From this observation and also from the data for short time intervals in Fig. 1, it can be concluded that the enhanced labelling of the supernatant-fraction RNA could not be due merely to a decrease in its supposed translocation to the microsomal fraction. Whatever the origin of microsomal RNA, it is evident that the lack of adrenal hormones affects the processes of cytoplasmic RNA synthesis at two separate points. That adrenalectomy also affects the synthesis of nuclear RNA is suggested by the results of Fig. 5, considered together with the fall in the amounts of uridine nucleotides (Tables 1 and 2); this fall in the pool of supposed precursors of nuclear RNA implies that the synthesis of this RNA is diminished more than is suggested by the slight diminution in the labelling of this RNA.

The decrease in microsomal labelling shows a striking parallelism, in its gradual onset after adrenalectomy, with the decreases in the incorporation of leucine into protein and in the amount of microsomal protein (Fig. 6). Since the rise in the labelling of supernatant-fraction RNA is much earlier, it is probable that at least some of this RNA, unlike the RNA in the microsomal fraction, is not directly concerned in protein synthesis. Admittedly it has not been proved in the present experiments that the small reduction in leucine incorporation truly signifies a fall in protein synthesis; but the absence of a significant effect of adrenalectomy on the pool of leucine in liver (Awapara, Skellenger & Manz, 1955) or on amino acid absorption by the liver (Awapara & Kit, 1954) argues in favour of this interpretation.

'Microsomal' material as prepared in some laboratories comprises both the microsomal fraction and the ultracentrifugal fraction as now prepared. The divergence between these fractions observed by Reid et al. (1956), with respect to the change in the incorporation of orotic acid after adrenalectomy, has now been confirmed (Figs. 1 and 2). A similar divergence has been observed by Jardetzky & Barnum (1956) in a study of RNA formation in regenerating liver.

SUMMARY

1. Adrenalectomy leads eventually to a marked decrease (exceeding 50%) in the levels, in rat liver, of uridine 5'-phosphate, uridine 5'-diphosphate (-pyrophosphate) and its glucurone acid derivative, and uridine triphosphate There is also some decrease in uridine diphosphate acetylglucosamine

The muscle was immediately immersed in cold saline when it was excised. After it was weighed, the cold tissue was homogenized in 0.5N perchloric acid. The neutralized supernatant was then placed on a simple, formate, ion-exchange hand column (Fig. 1). After this sample was placed on a column prepared from Dowex X-10, 200 to 400 mesh resin, the solutions listed in Table I were added in succession and 5 ml. fractions collected separately. Optical densities at 260 and 275mμ were determined, utilizing a model DU Beckman spectrophotometer, from which quantitative estimates of nucleotide fractions were made. In 2 of the samples, the adenosine nucleotide fractions were lyophilized, separately replaced on columns, and the eluents shown in Table II were passed through each (Figs. 2, 3, and 4).
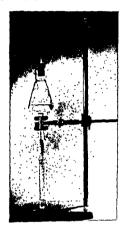
Fig. 1.—The simple hand column which was used in the study.

TABLE II. AMMONIUM FORMATE SYSTEM

NH₄ COOH M	NUMBER OF TUBES	NH₄ COOH M	NUMBER OF TUBES	NH₄ COOH M	NUMBER OF TUBES
0	4	0.40	5	0.75	5
0 05	4	0.45	5	0.80	5
0 15	4	0.50	7	0.85	5
0 20	4	0 55	7	0 90	5
0 23	4	0.60	5	1.0	4
0.25	4	0 63	5	1 2	5
0 30	4	0.65	5	1.3	7
0 35	5	0 67	5	1.5	4
0 38	5	0.70	5	2 0	3

NUCLEOTIDE LEVELS IN HUMAN CARDIAC MUSCLE

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THE chemical events during contraction of cardiac muscle are of sufficient interest to justify inquiry into the quantitative relationships of those compounds involved in transfers of energy associated with contraction. In this connection the determination of nucleotides in human muscle would seem to provide especially useful information, and a series of studies have been carried out to obtain the desired data. When auricular muscle was obtained at the time of cardiotomy and fractions of homogenate were separated by ion-exchange columns, it was found that sufficient quantities of nucleotides were recovered to yield a fairly consistent quantitative picture. The results obtained with samples from 10 hearts are reported.

METHODS

The principles of anion exchange chromatography described by Cohn[1] and the system devised by Hurlbert and associates[2] and Potter and associates[3] were utilized in separating nucleotides from acid extracts of fresh, homogenized, human myocardium. Samples were obtained from 9 female patients at the time of valvotomy for mitral stenosis and from 1 (Sample 3) with mitral insufficiency. The average age of the patients was 35 years, with ages ranging from 23 to 45 years. Seven of the patients (Samples 1, 2, 3, 6, 7, 8, and 9) were digitalized at the time of surgery and 2 were fibrillating (Samples 1 and 6). Curare and Pentothal Sodium were administered to the first 8 patients and cyclopropane and ether to the last 2. One patient (Sample 3) died on the third day after a pericardial sling was inserted for the treatment of mitral insufficiency. The remaining patients left the hospital in good condition.

TABLE I. FORMIC ACID SYSTEM

H COOH N	NH₄ COOH M	NUMBER OF TUBES	H COOH N	NH₄ COOH M	NUMBER OF TUBES
0 005	0 0	5	2 2	0 0	3
0 05	0 0	6	2.5	0 0	5
0 2	0 0	8	3 0	0 0	6
0 6	0 0	3	4 0	0 0	3
1 0	0 0	3	4 0	0 05	6
1 5	0.0	7	4.0	0 15	4
1 7	0 0	7	4 0	0 3	6
2.0	0 0	3	4 0	2.0	5

RESULTS

The average, total amount of acid-soluble nucleotides extracted was 25.6 ± 1.7 M × 10⁻⁴ per gram wet weight of auricular muscle. The appearance of the graphs of optical densities may be found in Figs. 2, 3, and 4, and the amounts of adenosine nucleotides determined are recorded in Table III. The adenosine nucleotide fractions were identified by using the two systems of eluents (Tables I and II) and comparing the position of each fraction to that of known samples of adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) obtained from commercial sources. The average amount
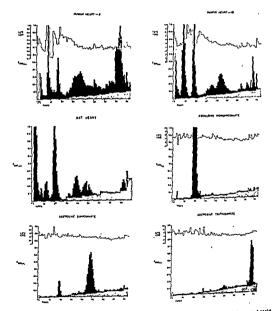
Fig. 3 —Spectrophotometer readings (E₂₆₀/ml) of samples from homogenates of human hearts (9-10), myocardium of the rat, and known samples of the adenosine nucleotides from yeast. The sample of ADP is contaminated considerably with AMP. Eluents were those of the formic acid system
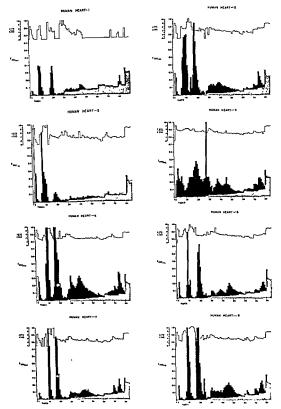
Fig 2 —The spectrophotometer reading (E_{260}/ml) of samples from homogenates of human hearts (1-8)   Eluents were those of the formic acid system   Shaded area represents the absorption due to formic acid and ammonium formate   The F fraction is the density of aqueous washings of the prepared column, and the S fraction is the density of the initial liquid recovered when the sample is placed on the column   The ratio E_{272}/E_{260} is approximately 0 4 for adenosine nucleotides, 0 6 for uridin nucleotides, 0 75 for guanosine nucleotides, and 1 to 2 for cytosine nucleotides.[1-4]

prominent role of adenosine nucleotides in phosphate-bond exchange of energy during contraction of the human myocardium and demonstrate the feasibility of direct investigations on fresh muscle in man as a supplement to indirect methods and studies on animals.
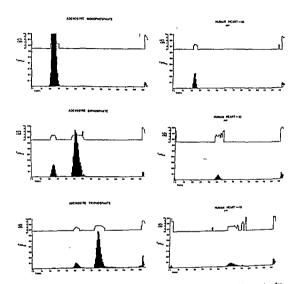
Fig 4.—The lyophilized fractions of AMP, ADP, and ATP from the formic acid-ammonium formate separations of Sample 10 were placed on columns and recovered by means of the ammonium formate system   They are compared to known samples of AMP, ADP, and ATP from commercial sources in this figure   The commercial preparation of ADP also contains AMP, and that of ATP contains both AMP and ADP.

CONCLUSIONS

1. Acid-so'uble nucleotides are present in human cardiac muscle in quantity sufficient for measurement in the amount of myocardium provided by an auricular biopsy.

2. The distribution of these fractions is similar for the myocardium of man, dog, and rat.

of AMP found was 6.6 ± 0.8 M × 10⁻⁴ per gram; the average quantity of ADP was 3.3 ± 0.4 M × 10⁻⁴ per gram; and the mean value of ATP was 2.8 ± 0.6 M × 10⁻⁴ per gram wet weight.

DISCUSSION

Peaks of absorption for the eluate were fairly constant in relation to constitution of eluent, and relative concentration of various nucleotides was consistent enough to justify confidence in the reliability of the procedure. The fractions from human myocardium were similar in position to those obtained when optical densities of fractions from the ventricle of the rat and dog[1] and auricle of the dog were determined, but differences in constitution were noted when these values for the myocardium were compared to other tissues such as liver.[2] No correlation was found between the amount of specific nucleotides in various samples and the age of the patient from whom the tissue was taken, the presence of fibrillation, or the administration of digitalis. The fact that the sample from the only patient expiring in the postoperative period also contained the smallest amount of ATP is interesting. The ratio ATP:ADP:AMP in the study was 1.0:1.2:2.4, whereas ATP was more abundant (2.6 times) than AMP in similar studies on canine auricular muscle. Also, the content of adenosine nucleotides in the canine ventricle was approximately 3 times that in the auricle. An advantage of the gradient elution[5] system used is that it reveals a spectrum of acid-soluble nucleotides rather than isolating selected compounds, and examination of Figs. 2 and 3 verifies the presence of nucleotides other than the adenosine system. Cytosine, uridin, and possibly guanosine nucleotides are probably among those present. Functions similar to those of the adenosine nucleotides would seem a reasonable assumption. Recent evidence that the enthalpy change of ATP hydrolysis is much smaller than previously reported[6] gives added significance to the presence of other systems which may contribute to the energetics of contraction. Also, they may not only act as coenzymes but may also contribute to protein synthesis as well. The results obtained (Table III) confirm the presumed,

TABLE III   NUCLEOTIDE CONTENT OF HUMAN CARDIAC MUSCLE

HEART NUMBER	SAMPLE WEIGHT (GRAMS)	AMP*	ADP*	ATP*	TOTAL NUCLEOTIDE*
1	0 530	5 83	2 62	1 63	23 45
2	1 655	6 90	3 02	1 58	22 25
3	0 631	2 50	0 36	0 00	19 58
4	2 251	4 44	2 00	1 07	20 54
5	2 694	10 02	4 09	1 44	25 11
6	0 926	8 09	4 08	1 62	27 82
7	0 947	9 58	3 99	2 18	28 77
8	2.084	10 29	3 24	0 11	25 18
9	1 636	3 64	5 69	13 22	39 30
10	1 947	4 81	4 25	1 99	24 22
Mean	1 530	6 60±0 84	3 33±0 44	2 76±0 61	25 62±1 63

*M × 10⁻⁴ per gram wet weight.

DEOXYRIBONUCLEOPROTEIN,
A GENETIC MATERIAL

By J. A. V. BUTLER and P. F. DAVISON, *London, England*

CONTENTS

I. Introduction

The suspicion that deoxyribonucleoprotein (DNA) may be the carrier of genic information was originally based on its occurrence in the nucleus alone, apparently as a structural element of the chromosomes. It has been strongly reinforced by the isolation of the transforming principles, consisting almost solely of DNA, and by the study of certain viruses, such as bacteriophages, of which DNA is an important constituent. As the subject has been reviewed several times in recent years (1-3), the present report will not attempt to give a comprehensive account of the field, but to supplement previous articles with points emerging from recent work.

II. Deoxyribonucleoproteins: Isolation and Properties

The isolation of DNP from mammalian cells depends primarily on the fact that it is usually insoluble and aggregated by dilute salt

3. The average amounts of adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate were 6.6, 3.3, and 2.8 M $\times$ 10^{-4} per gram, respectively.

4. A number of other nucleotides were also present in the samples. This suggests that the adenosine system is not the only one involved in protein synthesis and energy transfers responsible for contraction of the human myocardium.

REFERENCES

1. Cohn, W. E.: Ann. N.Y. Acad. Sc. 57:204, 1953.
2. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R.: J. Biol. Chem. 209:23, 1954.
3. Schmitz, H., Hurlbert, R. B., and Potter, V. R.: J. Biol. Chem. 209:41, 1954.
4 Burdette, W. J.: Am. Heart J. 52:193, 1956.
5. Tiselius, A.: Endeavour 11:5, 1952.
6. Podolsky, R. J., and Morales, M. F.: J. Biol Chem. 218:945, 1956.

ribonucleoproteins.   A number of subsequent procedures are possible, all of which have been extensively employed.

Extraction with Strong Salt Solutions. These methods, first used by Mirsky and Pollister (13), depend on the fact that the DNP is dissociated by strong salt solutions, and thereby dissolved. The extent of the dissociation depends on the salt concentration but it is regarded as complete in 2 M-NaCl and above (14) The DNP can be recovered, though not in its original form, by dilution. The DNA can be precipitated from the salt solution by the addition of alcohol, or the bulk of the protein can be precipitated by increasing the salt concentration almost to saturation (15).

Anionic Detergent Method (Kay et al. (16)). Anionic detergents like sodium dodecyl sulfate displace the basic proteins from their combination with DNA, and precipitate them. The DNA can then be precipitated by alcohol or, after dialysis, obtained by freeze-drying.

Enzyme Method (Butler, James, and Conway (17)). In this process the DNP is dissolved in 1 N NaCl and is then digested with a proteolytic enzyme for 2-3 days ̄ ̄ ̄ ̄   ̄ ̄ ̄ ̄ ̄ ̄ ̄ ̄ The cipit protein content than that usually obtained by the detergent method. A possible weakness is the opportunity for degradation by DNAase which might be present in the homogenate or is present in traces in the chymotrypsin; but the products are at least as highly polymerized as those obtained by the detergent method.

Sevag Process (18). The nucleoprotein is shaken with a mixture of chloroform and water (with some octanol) and the protein denatured and rendered insoluble at the interfaces. DNA with comparatively small quantities of protein can be extracted from the precipitate by extraction with salt solutions of various concentrations. It has been shown that fractionation of the DNA both with respect to composition (base ratios) and physical properties occurs in this procedure (see below). This process is also usually inefficient (19) in that only a part of the DNA, depending on the extraction conditions, is obtained.

Other Methods. With bacteria the separation of DNP and RNP cannot be achieved by these processes. Jones (20) with Mycobacterium phlei disintegrated the bacteria and precipitated the nucleoproteins with cetyltrimethylammonium bromide. The product contained ribonucleic acid (RNA), which could be removed by the charcoal method of Chargaff and Zamenhof (21) as modified by Dutta, Jones, and Stacey (22). Another method, employed by Hunter and Butler (23) with Bacillus megatherium, is to treat the disintegrated bacteria with sodium

Another method is an adaptation of the phenol method of preparing RNA from ribonucleoproteins. When cell homogenates are shaken with a phenol-water two-phase suspension, the RNA is normally found in the aqueous layer, and can be removed (24). It has been found that the DNA is similarly liberated into the aqueous layer when certain salts (e.g., thiocyanate) and other substances are present (25).

solutions, whereas the ribonucleoproteins remain in a dispersed form and can be removed by repeated washings with isotonic saline. It is helpful to isolate the nuclei in the first instance; but on a large scale this is a difficult operation in aqueous media and most preparative work has been carried out on DNP extracted from the isotonic saline precipitate. It usually can be separated from much of the cell debris by taking the upper layer of the centrifuged product. On dialysis with water the first product swells up into a gelatinous mass. Although attempts have been made to characterize the isolated "nucleoprotein" particle (4), it is doubtful if a native particle has been obtained. The native gelatinous substance is difficult to obtain in a molecularly dispersed form (5). On standing at room temperature some part of it becomes dispersed; but it has been suggested (5) (see below) that this is at least partly the result of enzyme action.

In certain fish sperm cells the DNA is combined with protamines (see review by Felix (6)) but in the somatic tissues the protein is the less basic type, known as histone, in which approximately 25% of the amino acid residues are of a basic character. It has been denied that nucleohistone occurs as such in nuclei (7); but a study of the composition of the nucleohistone isolated from thymus glands, as described above, by Davison and Butler (8), shows that it is of very constant composition and the total basic amino acids in the protein are not greatly less than the total amount of phosphate of the nucleic acid. It is very unlikely that this would be the case if their association is fortuitous. The structure of the nucleoprotein is discussed below.

It has been reported (9–12) that nucleohistones can be obtained in nonfibrous as well as fibrous forms. The former are obtained after repeated extraction of the nuclei with isotonic saline and it is suggested that something is extracted which is responsible for the high degree of aggregation of the nucleoprotein in the fibrous form. The relation between these forms has not been fully clarified.

III. Deoxyribonucleic Acid

A. PREPARATIVE METHODS

In view of the great variability of the product obtained, the method of preparation must be specified. The first step in all preparations consists in washing away soluble substances and, as far as possible,

C. DIMENSIONS AND PHYSICAL CHARACTERISTICS OF THE PARTICLES

The physical properties of dilute solutions of DNA (viscosity, flow birefringence, asymmetry of light scattering) suggest that the particles are highly asymmetric. The Crick and Watson structure required elongated fibers of diameter about 20 A. Fibers of the expected dimensions have been observed in the electron microscope (31).

A wide variety of molecular weights has been found. The earlier values (35) based on sedimentation and viscosity or diffusion (1-2 × 10⁶) are probably too low since the solutions used were too concentrated for a proper extrapolation to zero concentration. More accurate values can be obtained either from sedimentation and viscosity measured at very low concentrations (36) or from light scattering (37). The values most frequently obtained are of the order 6-8 × 10⁶, but appreciably lower and higher values are often observed. The significance of these is discussed below.

A molecular weight of 6 × 10⁶ implies in the Crick and Watson model a length of about 3 × 10⁴ A. The actual lengths observed by physical measurements are all appreciably less than this (from 2-5 × 10³ A. (37)). It therefore appears that, although its basic structure may be in accordance with Watson and Crick, the particle is not a simple straight rod

Salts have a marked effect on the viscosity of solutions. However, it was shown by Sadron and Pouyet (38), and confirmed by Conway and Butler (39), that the effect of salts disappears or at least becomes comparatively small at very low concentrations. This means that the effect of salts is not primarily on the form of the isolated DNA particle but on the interactions between them. It is deduced from this that the particles must be comparatively rigid. The interactions which occur in aqueous solution, since they are greatly reduced by small concentrations of salts, must be of an electrostatic character (40). However, the theory of such interactions is not well established, although a similar phenomenon has been observed in some cases with synthetic polyelectrolytes (41). A number of investigations have been made of the titration curves of nucleic acids, which are in good agreement with the requirements of the structure (42). Cox and Peacocke after a careful analysis of the curves found no indication of appreciable amounts of triply esterified phosphate (43).

When prepared from tumors, by methods which with normal mammalian tissues give a good product, it is found that the DNA obtained is contaminated with RNA (26). This does not appear to be bound in any way to the DNA, and is removable by the action of ribonuclease or by dilute alkali; but it is not completely removed by washing the mixed nucleoproteins with isotonic saline. The incomplete separation of the two types of nucleoprotein from tumor homogenates may be due to the abnormal behavior of the ribonucleoproteins which have been shown, in some instances, to have an abnormal composition.

B. COMPOSITION AND STRUCTURE

The most stimulating contribution in recent years has been the structure proposed by Watson and Crick (27), which not only accounts for many of the physical characteristics, and is with some modification in accordance with the x-ray diffraction patterns (28,29) (although it is not possible to derive any unique structure from these), but also suggests a way in which the reduplication of nucleotide threads with bases in a specific order can take place. It is unnecessary to describe the structure in detail, since numerous accounts are available, but it will be recalled that adenine is paired uniquely with thymine and guanine with cytosine. This accounts for the ratios of the bases observed in many analyses of DNA, although it is to be noted that cases in which the ratios differ from unity by an amount which is greater than the experimental error continue to be reported (31).

It has been suggested that other types of pairing are possible, e.g., adenine with guanine and thymine with cytosine; but no evidence has been obtained that such pairs actually occur.

It has, however, been found that certain bases can be replaced to some extent by substituted derivatives having a similar basic structure (i.e., —OH and —NH₂ in the same positions). Thus part of the thymine is replaced by 5-bromouracyl when several microorganisms are grown in the presence of this substance (32) and a similar replacement may occur in bacteriophage. 5-Hydroxymethylcytosine also occurs naturally in place of cytosine in the DNA's of T2, T4, and T6 bacteriophage (33).

kinetic studies and must remain open for the present. Heating with acid or alkali naturally causes a much greater disruption of the DNA structure (47, 50) owing to hydrolysis of the nucleotide links and other effects.

The action of urea has been studied with contradictory results. Conway and Butler (52) found that treating DNA with concentrated urea solutions produced permanent changes; a decrease of molecular weight by a factor of 2 was also indicated, but it was uncertain if this was significant. More recently Conway (53) has found that the intrinsic viscosity of DNA in urea is markedly influenced by the salt concentration, indicating that the particle is more flexible in the presence of urea. The intrinsic viscosity was greater in urea than in water, but at moderate concentrations of DNA it was found (49) that urea affects the viscosity at zero shear rate in a manner similar to salts, while Doty (54) denies that urea has any effect on the viscosity of DNA. The difference of these findings is probably due to a deviation in behavior at different shear rates.

Since urea is a good hydrogen bond breaker it might be possible for it to disrupt the hydrogen bonding between the twin nucleotide threads. Here again there is controversy as to what actually occurs. Alexander and Stacey (55) claimed that, with herring sperm DNA, the molecular weight as determined by light scattering was reduced to one-half in the presence of urea, while no such effect occurred with thymus DNA. They interpreted the former results as indicating that urea split the DNA twin helix into two halves, while the latter result was explained as due to a greater stability caused by the pressure of metal ions or proteins (56). Doty (54) has claimed, however, that no change of molecular weight and no other evidence of denaturation were observed in either case. It is found that the presence of urea decreases the temperature at which denaturation by heating occurs.

E. HETEROGENEITY

The exact evaluation of the physical characteristics involves a prior knowledge of the heterogeneity of the material. In the earlier work on the electrophoretic mobility and sedimentation very sharp boundaries were observed, but it is now known that these are not true indications of homogeneity but are the consequence of a marked concentration-dependence of the property in question. The use of

Mathieson and McLaren (44) have made fresh determinations of the
electric charge on the particle at various pH values.

D. EFFECTS OF HEAT AND REAGENTS

When DNA solutions are heated for only short periods at 80° or
above, the viscosity falls very greatly (45).   The fall in viscosity is
accompanied by a rise in the optical absorption coefficient (46).   The
temperature at which the fall of viscosity begins is fairly sharply de-
fined, *i.e.*, the temperature coefficient is very high, as is expected for a
cooperative phenomenon in which a number of bonds must be broken
in order to bring about the physical change observed.   The effect of
heat is therefore attributed to the breakage of the hydrogen-bonded
structure of the intact double nucleotide thread (47).   It has been
shown by Cox and Peacocke (48) that, after heating, the groups which
are hydrogen-bonded in the intact DNA become accessible to titra-
tion, and the amount of breakdown of the hydrogen-bonded struc-
ture can be deduced from the change in the initial titration curve

There has been some divergence of opinion on the question of
whether the molecular weight is reduced by heating.   Dekker and
Schachman (49) found that, simultaneously with the decrease in
viscosity produced by heating to 100° for 15 minutes, the sedimenta-
tion constant fell from 20 to 6   This could be interpreted only
as the result of a decrease in molecular weight by a factor of *ca.* 100.
Dekker and Schachman therefore suggested that the nucleotide
threads of the DNA consisted of comparatively short overlapping
segments   This result referred to the heating of DNA in water alone;
in the presence of salt the changes of molecular weight are much
smaller.   Doty and Rice (50) have claimed that under this condition
no change of molecular weight occurs; however, Shooter, Pain, and
Butler (51) found that some decrease of molecular weight (by a
factor of 4–6) occurred on heating for short periods at 100°.   There
is no doubt that in the absence of added salt a considerable amount of
degradation occurs on heating at 100°.   The question arises whether
this is due to the presence of interruptions originally present in the
nucleotide chains or to the hydrolysis of nucleotide linkages on heating
with water.   There is no doubt that the amount of degradation ob-
served increases slowly with time, but there is no evidence that an
appreciable hydrolysis detectable by chemical analysis occurs even in
long periods of time.   The matter can be decided only by careful

characteristics of DNA during various stages of preparation (58) These can be observed at the nucleoprotein stage by adding sufficient salt (2.5 M) to dissociate it.   The characteristics of the DNA in this solution are practically the same as those of DNA subsequently isolated from the same solution by the detergent method.   However, when the native nucleoprotein is kept in its gel-like condition, a slow degradation of the DNA in it occurs; this can mean only that a DNAase or similar agent is present in the nucleoprotein gel.

F. FRACTIONATION OF DNA

It was shown by Chargaff et al. (63) that the extraction of nucleoprotein gels, which had been denatured by shaking with chloroform as in the Sevag process, with salt solutions of increasing concentration resulted in fractions of differing composition, the fractions obtained with the greater salt concentrations being relatively richer in adenine and thymine.   These authors concluded that the bonds between the denatured protein and the different bases were of different strengths and that those with adenine/thymine required a greater concentration of salt.   Lucy and Butler (64), however, found that a similar effect could be obtained by successive extractions with a salt solution of constant concentration.   It was also found (65) that the proteins present in the DNA fractions extracted in this way varied in composition, the first extracts containing a marked excess of lysinerich histone.

However, it cannot be inferred from this that the guanine-rich fractions are preferentially associated with the lysine-rich histones, as the two processes of fractionation of the DNA and of the proteins may be occurring simultaneously (66).

Chromatographic methods of fractionation on columns have also been devised.   G. L. Brown has developed a column in which denatured histone is supported on kieselguhr (67), which gives fractions having somewhat different base ratios.   This has been applied in conjunction with the "star" method to isolate the large size particles of DNA present in bacteriophage.   A chromatographic separation yielding a large number of fractions, with Ecteola resin, has been developed by Bendich (68).

Other methods involve fractional dissociation of complexes of DNA with polylysine (69) and with polysulfates (70).

optical absorption methods of determining the concentration in the boundary permits the observation of the behavior at very much lower concentrations than is possible with methods depending on the refractive index.

When studied by this method at concentrations below 0.005%, in the ultracentrifuge, a marked heterogeneity of the sedimentation constant is observed (57). There might be some doubt whether the boundary is completely stable at such small concentrations, but careful tests of stability show that the boundary is completely stable and that diffusion during the run is negligible. The reality of the heterogeneity has been confirmed by centrifugation on a preparative scale (58).

Since such variations of sedimentation constants cannot be accounted for as due to the variation of length of a linear rod-shaped particle (59), it is concluded that heterogeneity of shape as well as of mass occurs. This could happen by the aggregation of DNA particles laterally or in some kind of network. Such aggregation might be brought about by crosslinks between DNA fibers produced by, e.g., divalent metal ions, or by protein. The former does not seem to be important, since neither the addition of divalent metal ions, nor of complexing agents (Versene), has much effect on the form of the distribution curve. However, DNA, as prepared by the detergent method, contains residual quantities of protein (up to 1%) of a non-histone character. That this protein is responsible for at least part of the heterogeneity is shown by the fact that chymotrypsin greatly reduces the proportion of the material having very high sedimentation constants, and brings the distribution curve down to the region of those of DNA produced by the "enzyme" method (60). It is obvious that small residual quantities of protein have a considerable effect on the physical properties of DNA and little credence can be given to the particle mass and dimensions until this effect is eliminated. It is to be noted, however, that even after removal of protein by proteolytic enzyme a considerable spread of sedimentation constant remains and it is probable that there are other variable factors arising from the method of preparation. DNA from bacteriophage shows a smaller spread of sedimentation distribution constants than that from mammalian tissues (61); and a preparation of Pneumono-coccus-transforming principle was also relatively homogeneous (62).

A study has also been made of the changes of the sedimentation

developed an alternative chromatographic procedure, using the carboxymethyl cellulose of Sober and Peterson (85). Further experiments have shown that all the histones can be eluted, from this adsorbent, and at least three components can be detected and partially resolved (87).

The task of isolating homogeneous protein species from the aggregating histone mixture may be formidable, and it may ultimately be necessary to deaminate the proteins, or otherwise block the basic groups.

(2) Characteristics of the Basic Proteins

Studies on the chromatography of the calf thymus histones by Crampton, and by Davison, have raised two points of importance. Crampton found that on occasions a component which had been resolved chromatographically as a single peak, when rechromatographed appeared to be complex, one fraction running through the column with the front and the other appearing in the same position as it ran before (86). Davison (87) has also observed a similar phenomenon, and noted that the fast running fraction can be eluted from IRC-50 with sodium chloride, whereas the histones can be displaced only with barium ions. These observations suggest that the eluted proteins have become degraded during recovery, and confirm the findings of Butler et al. (88), who reported the ready degradation of the histones in neutral solutions. The fact that Crampton, as mentioned above, found the chromatographic behavior of the histones simplified when his preparation at neutral pH was completed more rapidly also points to the dangers of proteolysis It may therefore be questioned whether the salt–ethanol method of preparation (88–91), is really as "mild" as has been suggested. The similar doubt will apply to the preparative method used by Desreux and Ott, who have removed the dissociated DNA from the proteins by fractional centrifugation in strong salt (91a).

On the other hand, the use of strong acids may be reasonably criticized in view of the lability of certain peptide bonds. $N/10$ mineral acid has been most generally used to extract the histones from the isolated nuclei, nucleoprotein, or "Sevag gels." It will be shown below, however, that at this strength the histones (and, as Stedman and Stedman have reported, the protamines (92) are not completely extracted. Toennies et al. (93) reported the complete extraction of

IV. The Proteins Associated with DNA

A. BASIC PROTEINS

(1) *Fractionation Methods*

The histones and protamines from specific tissues were for years treated as homogeneous materials and many careful analyses of, *e.g.*, calf thymus histones, have been made. A critical inspection of these analyses, however, shows impossibly wide differences between the data of various authors. It became increasingly plain that these histones were a mixture of proteins, and from this mixture different workers were selecting different fractions In 1950 Stedman and Stedman (71) demonstrated a partial fractionation of the calf thymus histones and those from several other tissues. Since then the demonstrations of heterogeneity have been repeatedly made by electrophoresis (72–74), by ultracentrifugation (72,75,76), by fractional precipitation (72) and extraction (77,78), and by chromatography (79,80).

As early as 1929 Felix and Dirr (81) showed that one of the protamines, clupeine, was a mixture of proteins. Since that date a variety of techniques has been applied to determine the complexity of the protamines, the most powerful being countercurrent chromatography (82) and chromatography on activated alumina (83). The fractions isolated by these two methods differ in composition.

Among the histones, fractionation has been complicated by their tendency to aggregate. Despite this, from three laboratories almost simultaneously reports were made of the isolation of a lysine-rich histone from calf thymus (77–79). Although the fractionation methods differed widely, the analyses agreed sufficiently closely to suggest that a single protein had been obtained, largely freed from contaminants. From physical studies a molecular weight of 18,000 was proposed (72).

The most promising method of fractionation would appear to be chromatography. Crampton, Moore, and Stein, using the ion-exchange resin IRC-50, succeeded in resolving three components from various calf histone preparations, but they could recover only about 50% of the protein they applied to the column. More recently Crampton (84) substantially improved the recovery by extracting the histones more rapidly, and at the same time the complexity of the elution pattern was diminished. Davison and Shooter (80) have

It may be argued that several proteins (e.g., hemoglobin) have an abnormally low molecular weight in strong urea solutions, but, since it is unlikely that urea is splitting peptide bonds, and in view of the postulated structure of nucleoprotein (30), the most fundamental point of interest is the length and polydispersity of the individual peptide chains.

Terminal amino acid and sequence studies have provided some valuable information on the structure of the protamines (6), although Seanes and Tozer found N terminal alanine in addition to the proline found by Felix (83). Studies of the histones with the fluorodinitrobenzene method of Sanger were made recently by Haley (94), who detected only a limited number of end groups: valine, leucine, glycine, alanine. By using carboxypeptidase he found alanine, leucine, valine, and tyrosine as carboxy terminal groups. He obtained only a partial disproportionation of these end groups when the histones were fractionated in the ultracentrifuge into the "light" and "heavy" components but that is not surprising, because the "heavy" histone is an aggregate which probably includes some of the proteins present in the "light" fraction. It is important to note that the yield of terminal groups from a known weight of histone indicated a number-average molecular weight of 7,000 to 9,000. This figure agrees well with that determined by Crampton by ultracentrifugation in urea. It should be borne in mind, however, that the presence of a small proportion of low molecular weight polypeptides would outweigh the longer chains in such a calculation of number-average molecular weight. This word of caution is necessary because Phillips (95) has found proline, alanine, serine, and lysine as N terminal residues, but the yield, even when the coupling was carried out in the presence of guanidine, never indicated a number-average molecular weight less than 30,000. Further work is undoubtedly necessary to decide between these conflicting results.

Recent investigations by Hamer (96) have confirmed the earlier reports by Hultin and Hearne (97) of the existence of basic proteins with a composition characteristic of neither protamines, with their enormous preponderance of arginine, nor histones, but of something between the two. These proteins, which were extracted from the sperm of sea urchins, starfish, and squid, could not be precipitated from solution with ammonia as can the arginine-rich histones.

histones from a "Sevag" gel with hydrochloric acid at pH 2.5, but Davison has found it necessary to employ $N/4$ or stronger acid, and even then the coagulated mass needed to be ground in a ball mill. Though these extractions are carried out at low temperatures, it has been found that the DNA is degraded (72) with the loss of some of the purines. There is also an undoubted risk that some of the amide nitrogen may be lost from the proteins (if nothing more) and this loss may introduce a spurious complexity to the extract. Few of the protamines contain the dicarboxylic acids so that the use of acids for their extractions is probably less dangerous. Equally, however, there has been no demonstration of proteolytic degradation of these proteins in neutral solutions.

It has been shown that the histones interact and aggregate to a marked degree (72,73). Davison and Shooter (80) have shown by physical studies that the aggregation of the histones is dependent on pH and temperature. Large aggregates are formed, particularly above pH 7, the sedimentation coefficients vary between 10 and 20 S, according to the condition under which the aggregation occurs. The proteins that sediment more slowly (1–2 S) appear to form complexes, the nature of which changes considerably as the pH is taken below 4.5. The existence of intermolecular interactions is not surprising in view of the relatively high content of the dicarboxylic acids in the basic proteins, but there is evidence that the bonds are not solely ionic, since the high molecular weight aggregates (10 S or greater) are not split in the presence of salt (80).

From physical studies on the whole and fractionated histones it has been estimated that the molecular weights lie over 18,000 (72,88). Crampton (86), however, has found that the lysine-rich and arginine-rich histones, separated by chromatography, have molecular weights of only 7,000 and 8,000, respectively, in 6 M urea. Shooter and Davison have confirmed a substantial drop in the sedimentation coefficient of the histones, when urea is added to the solution (87a). Since these molecular weights are lower than those calculated from the amino acid composition (the percentage of tyrosine reported in the lysine-rich histones indicates a minimum molecular weight of about 20,000), it suggests that these chromatographically homogeneous fractions are complexes containing proteins of differing composition. In view of the over-all agreement of the analyses it is probable that these complexes are of fairly specific composition.

uptake of isolated nucleoprotein, suggested that some of the phosphate groups were not blocked by histone. Dounce (111) also suggested this from observations on the contraction of nuclei with lowering of the pH. Davison and Butler (8) found there were only enough basic amino acids in calf thymus nucleoprotein to neutralize 85% of the phosphate groups. (In these analyses the total phosphorus of the nucleoprotein was taken as DNA phosphorus because the phospholipid content of these preparations is very small.) Whether these phosphate groups are free *in vivo*, or whether some component is selectively (and reproducibly) lost in the isolation procedure, is not known. Dye-binding experiments suggest that the free groups occur in sequences.

The nature of the protein attached to the DNA in mammalian sperm is at present uncertain. Dallam and Thomas (112) confirmed earlier reports that no nucleoprotein could be extracted from these sperm with water or strong salt solutions. By extracting the sperm with sodium hydroxide and isoelectrically precipitating the proteins of the extract they obtained a basic protein which they called a histone. They noted the high content of arginine in this fraction. Although the histones are, in fact, characterized by a preponderance of lysine, the lysine-rich components may not have been precipitated isoelectrically. Vendrely *et al.* (113,114) have presented partial analyses of isolated nuclei and nucleoprotein preparations. They have pointed out that the arginine/phosphorus ratios fall into two fairly distinct groups corresponding to the presence of either histones or protamines. They suggest that the protein in bull sperm is a protamine (114). The peculiarity in structure which makes these sperm so insoluble, in contrast to those of fish and birds, is at present unknown.

B. NONBASIC PROTEINS

In 1942 Mayer and Gulick (115) detected some nonhistone proteins in nuclei isolated by the Behrens technique. The Stedmans, in 1943, described a nonbasic protein from nuclei isolated in aqueous media (116). Since that time a number of workers, including Wang, Dallam, and Thomas and co-workers (112,117), Hamer (118), and Mirsky and Ris (119), have obtained proteins which they have distinguished from the histones. Strictly, the various proteins which may occur in different cell nuclei lie outside the scope of this review, which is

of malignant nuclei is usually greater than that of nuclei from nor-
mal cells (107).

Thus there is no certainty that all the proteins in the extracts from
isolated nuclei (and presumably from nucleoprotein) are nuclear in
origin. It is evident that a distinctive test for histones is needed,
and fortunately the reagent introduced by Mirsky and Pollister (13)
would appear to fulfil this need. This test may fail if it is applied to
degraded proteins as Bernstein and Mazia (108) have shown, but if
used with appreciation of its limitations the test provides a useful
means of differentiating between the histones and the many other
proteins from the cell which may contaminate the preparations. The
presence of tryptophan in the protein extracts has also been used
as a test for contaminants in the histones: most histones have been
shown to contain no tryptophan, and although some recent analyses
have still reported traces of this amino acid (e.g., Harper and Morris
(109), and Toennies et al. (93)) it is generally concluded that the
histones as a class contain none.

(4) Constitution of Nucleoproteins

Feughelman et al. (30) have proposed a model structure for nucleo-
protamine in which the polypeptide chain is arranged helically around
the twin spirals of DNA, with the arginine residues directed toward
the axis of the helix, successive residues being attached by salt bonds
to the phosphate groups of each DNA chain alternately. It was
postulated that the nonbasic amino acid residues, which Felix and
co-workers (6) have found to occur in pairs, formed small loops pro-
jecting out from the axis of the composite molecule. It was also
suggested that the nucleohistone structure might be similar.

The histones contain only 25% basic amino acid residues whereas
the protamines have more than 60%. Therefore any structure
similar to the nucleoprotamines must allow for an average of three
nonbasic residues separating each basic one, and such molecules
should have a considerably greater diameter (8). Amino acid se-
quence studies on peptides from the histones should show whether or
not the basic residues are arranged at regular intervals.

The x-ray diffraction studies give only the general impression of the
nulceoprotein structures, and the details must be investigated by
other means

Mirsky and His (110), from observations on the histone and dye

acid contain 27% histone (isolated as the sulfate). Mirsky and Pollister, from nucleoprotein with substantially the same percentage DNA (and, hence, presumably, the same proteins), have extracted 40% of the material as histone. Davison (87) has extracted, from nuclei isolated in the same way as Stedman (92) has described, 40% by weight of the nuclei as histone, by a single extraction with $N/4$ hydrochloric acid. Hamer (118), from the nucleoprotein extracted from thymus nuclei, could dissolve only out about half of the protein as histone. Davison et al. (72), from thymus nucleoprotein, extracted all but 8% of the protein as histone, and the extract under electrophoresis at pH 6.5 showed only cathodically migrating proteins. None of the protein could be precipitated by the "Mirsky" reagent (1.88 M sulfuric acid containing 0.34 M mercuric sulfate). From this incomplete survey it is obvious that the amount of non-histone protein any author will ascribe to a particular nucleus will vary inversely with his success in completing the extraction of the histones.

Mirsky and Ris (119) have shown that in strong saline nucleoprotein is dissolved with the liberation of insoluble protein fibrils which they have termed "residual chromosomes." This protein would appear to be identical with the tryptophan-containing protein (TrPr) Mirsky and Pollister (13) described earlier. It is also probable that the acid lipoprotein which Wang and co-workers (117) have extracted from isolated nuclei with dilute alkali, and at least a portion of the chromosomin described by Stedman and Stedman (116), are the same protein fraction.

Since strong salt dissociates the residual chromosome from the DNA, it is presumably attached by salt bonds. It is possible that there are other minor components linked to the DNA in other ways; Dounce and Monty (125) have attempted to remove the gelatinous protein left with the DNA when rat liver nuclei are extracted with acid: their lack of success prompted a search for some other bonds between the protein and DNA and preliminary results suggest that there may be some. Potter and Dounce have found phosphoamide bonds in ribonucleoprotein (126) and something similar may exist in DNA. Nevertheless Smillie et al. (91) have claimed that the whole of the protein in thymus nucleoprotein can be dissociated in 2 M salt: although the results from one tissue cannot be expected to apply to another, it remains a possibility that the firm attachment of this

concerned with the proteins bound to the DNA. It is still not entirely certain that the proteins found in extracted/deoxyribonucleoprotein (DNP) are actually bound to the DNA *in vivo*, although the x-ray diffraction examination of sperm (30) adds weight to the evidence previously cited (1) that suggests that the DNP is not an artifact. Dounce (120) has offered some puzzling experimental evidence which he interprets to mean that, within the nucleus, *in vivo*, the DNA is combined with proteins other than the histones. This uncertainty makes it difficult to decide the demarcation between nuclear and nucleo- proteins. However, this difficulty appears to be largely resolved by the fact that nuclei isolated by most aqueous procedures (and, here, thymus nuclei isolated in calcium–chloride sucrose must be expected) have a composition almost identical to DNP, or the isolated chromosomes of Mirsky and Ris (119).

The fact has been explicitly demonstrated by Felix, Fischer, and Krekels (6) in the case of certain sperm nuclei, and Hamer found that almost the whole of calf thymus nuclei can be dissolved in strong saline (118). It is also implied when the analyses quoted by various authors are compared· thus Mirsky and Pollister found the same percentage of DNA in thymus nuclei and isolated chromosomes, and the Stedmans reported the same figure for their nuclei (71). As the Stedmans have pointed out, this agreement would appear to identify the "chromosomes" with the whole nuclei. The Stedmans dismissed this as absurd, but the evidence since obtained to demonstrate that "aqueous" nuclei have lost a very considerable proportion of their protein content (121–124) has made their criticism invalid.

Except for the case of mammalian sperm, therefore, the "aqueous" nuclei and the DNP are almost identical in composition. The sperm are excluded because, up to the present, the extraction of an intact nucleoprotein with water or strong salt from this source has not been achieved.

The composition of the nuclei or DNP preparations has usually been studied by extracting each of the recognized major components selectively: any insoluble residue has then been referred to as a further fraction. Such a scheme may be satisfactory if none of the components becomes denatured or degraded, but the results deduced from such studies do not seem to justify the faith put in the method. Stedman has reported that calf thymus nuclei isolated in dilute acetic

Chargaff (130) and Butler et al. (11) have shown that all the protein-DNA bonds are not dissociated until the strength of the salt solution is taken above 2 5 M. The RNA also would appear to be rather loosely bound since it was not detected when TrPr was first identified. Khouvine et al. (103) have also reported a nonhistone protein in rat epithelioma nucleoprotein which was obtained by the method Mirsky and Pollister used in their early experiments, and this too contained no RNA.

Mirsky and Ris (110) have shown that the protein has an amino acid composition which distinguishes it from the cytoplasmic proteins. Many workers have commented upon the tryptophan content of this protein fraction, a feature distinguishing it from the basic proteins. Since it has been characterized mainly by its insolubility, however, there has been little attempt to assess its heterogeneity.

Another nonhistone protein in the DNP which is soluble in 10% saline has been reported by Mirsky and Ris (119), who found that, when the saline solution containing the dissociated nucleoprotein of liver or thymus was "Sevaged," not all the protein in the gel could be extracted with acid. Many authors have used this method to prepare basic and nonbasic proteins since then. If the DNP has not been clarified by high-speed centrifugation in strong salt, this gel will presumably include the residual chromosomes (e.g., Toennies et al. (93), but even if these have been removed it cannot be assumed that all the proteins which are not extracted by acid are nonhistone Butler et al. (88) and Smillie et al. (91) have found that denatured histones are insoluble in acid.

Recently Allfrey, Mirsky, and Osawa (105) reported a fractionation of thymus nuclei isolated in sucrose, which appeared to indicate the presence of at least four nonhistone proteins. Which of these is associated with the DNA is not certain. The authors identified none of the fractions with the residual chromosome. The justification for this scheme of fractionation remains to be demonstrated.

V. Genic Significance of Deoxyribonucleoprotein

A. THE MODE OF DUPLICATION OF DNA

In the Watson and Crick structure, the two separate halves are exactly complementary to each other. It was pointed out by them (131) that this provides a means of duplication in which the nucleo-

nonbasic protein fraction to the DNA may result from the denaturation of either component in acid solution. It is notable that most authors who have begun to fractionate the nuclei by extracting the basic proteins with acid have found the nonbasic protein very firmly bound to the DNA, but when the proteins and DNA have been extracted by strong salt solution, the residual protein has become less intractable.

Mirsky and Ris (127) found that the proportion of nonbasic protein (or residual chromosome) in their isolated chromosomes varied with the metabolic activity of the tissue of origin. They found that the residual chromosome constituted only 8% of the calf thymus chromosomes. Davison et al. (72) and Toennies et al. (93) reported a similar proportion of nonbasic protein in thymus nucleoprotein. The presence of a nonhistone protein in fish sperm is still debated. Felix and his school (6) have concluded that the nucleus of many specimens they have studied consists of protamine nucleate alone. This view has been opposed by Stedman and Stedman (101), who have pointed out that a nucleus so constituted contains no material to form the nuclear sap. Recent studies of snail sperm nuclei by Grassé and his co-workers (128), however, seem to suggest that the maturing sperm nucleus is a tightly packed mass of fibers (presumably chromatin). Mirsky and Ris (129) found that fish sperm contained a very small residual chromosome, and they detected 0 15% RNA in their trout nuclei. Felix et al. have not detected any RNA in fish sperm, but it is probable that this amount is not detectable by any method of analysis unless the fraction containing it is first concentrated. Felix and his co-workers do report finding a very small portion of the sperm nucleus to be insoluble in strong saline and they suggest this is part of the structure where the sperm tail enters the nucleus. This insoluble fraction may be identical to the residue described by Mirsky and Ris.

Bernstein and Mazia (108) and Hamer (96) found a considerable proportion of nonhistone protein in echinoderm sperm. The reason for the higher content of nonbasic protein in these sperm, when compared to fish sperm, is not clear, but if an explanation could be found it might explain the function of this protein.

Mirsky and Ris (129) have reported that the residual chromosomes contain both RNA and DNA. The presence of the latter may not have any great significance because both Crampton, Lipshitz, and

by their appearance. The resolving power of the radioautographic method is also insufficient to distinguish the radioactivity of individual chromosomes, but it is possible to obtain a measure of the total amount of radioactive material present in the cell and conclusions have to be derived from this information If chromosome reduplication results in the formation of one original chromosome and one completely new chromosome, there will be one chance in 2^6 (= 64) that one daughter cell will contain 6 completely new chromosomes and the other 6 old chromosomes. We shall therefore find in this proportion of cases cells which have either no radioactivity or a full amount; in other cases there will be inequalities of the amount of radioactivity in the two daughter cells, while on the Watson and Crick hypothesis all new cells will be equally radioactive. Mazia, using thymidine as the radioactive marker, has stated (135) that differences in the radioactivity of daughter cells are observed: which favour the first hypothesis.

Another favorable system for studying the duplication of DNA is bacteriophage, which has been studied by Levinthal (136), who has developed a method by which the radioactive content of individual particles of DNA can be determined. A radioactive bacteriophage (T2) is obtained by allowing multiplication for a considerable time in the presence of P^{32}. The radioactivity in the individual DNA particles obtained from this bacteriophage is measured by the size of the "star" around each particle when they are embedded in a photographic emulsion. Stars were obtained of a size equivalent of 40% of the activity of the DNA in the original bacteriophage unit. When allowed to multiply in a nonradioactive bacterium, it was found that all the first generation progeny gave stars equivalent to about 24% of the original activity. This indicates that a sharing of radioactivity had occurred in duplication, as is to be expected from Crick and Watson's hypothesis. A similar conclusion has been drawn by Stent (137), who estimated the amount of P^{32} from the fact that the emission of radiation by P^{32} kills the phage particle.

B. FUNCTION OF DNA

If DNA is the genic material in that it carries in some sort of code the genetic information, it is natural to ask how it operates. One of the most important functions occurring in cells is the synthesis of highly specific proteins and it has been tempting to think of DNA

tide thread can be reproduced with its bases in any specific order, since each half of the structure when separated is capable of being completed in one way only. If this is the method by which reproduction of the DNA threads occurs in cells, it would follow that, when the duplication of a DNA particle occurs, each new particle will consist of one-half of old material and one-half of newly synthesized material. This is the simplest mode of reproduction, although it has difficulties. For example, it has been pointed out that, if the nucleotide threads are coiled into a spiral (one turn for each ten nucleotide pairs), the process suggested necessitates a complete uncoiling of the spiral, *i.e.*, about 500 turns for the whole of an average DNA particle. Ways in which the uncoiling could be effected have been suggested (132), and a study of the energetic aspects (133) suggests that the difficulties involved may not be too formidable. On the other hand it is certainly possible to devise schemes in which the essentials of the Watson and Crick scheme are retained but the original double thread remains unaltered at the end of the process (134). If any such process occurred the result would be an unaltered original DNA particle and a completely new freshly synthesized one.

To distinguish between these possibilities, experiments have been made using radioactive substances which can be incorporated into the newly synthesized DNA. It is fortunate that when no DNA synthesis is occurring, very little exchange even of phosphate occurs with intact DNA. In order to carry out such experiments, it is necessary to be able at least to distinguish unaltered DNA particles from those which have undergone duplication. In mammalian tissues the matter is complicated by the fact that the chromosome, which acts as a unit in cell duplication, contains a very large number of DNA particles. Is it to be expected that, when the chromosome is duplicated, all the DNA particles of each of the daughter chromosomes are similarly related to that in the parent chromosome? If this is not the case, no information can be deduced about the mode of duplication of the DNA particles from the distribution of newly synthesized material between chromosomes.

Experiments of this kind have been carried out by Mazia (135) using cells of the plant *Crepis*, in which it is possible to distinguish for a time when cell division has taken place, the two daughter cells derived from one parent. There is, however, the complication that each cell has 6 chromosomes which cannot be readily distinguished

The progress of change from histone to protamine in spermatogenesis has been clearly demonstrated by Alfert (142) using a differential staining technique. The replacement of the histones occurred at a late stage of spermatogenesis

It might be possible to correlate the nature of the histones with differentiation during development. Not enough is known at present about the character of histones to establish whether tissue specificity occurs (see p. 174) apart from the difference of sperm and somatic tissues.

It may also be significant that the total basic groups available in the histone is less than the number of phosphates of the DNA, leaving about 15% free phosphate in thymus nucleoproteins and 6% free phosphate in sperm. On the hypothesis of the Stedmans (143) that the histones and protamines act as gene inhibitors, the free phosphate groups would be present on the metabolically active parts of the DNA. However Allfrey, Mirsky, and Osawa (105) found that the blocking of these groups by a variety of substances did not diminish the synthetic activity of the nucleus cell. On this hypothesis the number of distinct histones required would be equal to the number of genes, and, further, some mechanism would have to be envisaged for covering or uncovering the genes at the right stage.

Section added in proof. Since completing the review, some further observations which deserve mention, have come to our notice. J. Murray Luck *et al.* (144) have developed a method of chromatographing histones on Amberlite IRC-50 columns, using increasing concentrations of guanidinium chloride as the eluting agent. A large number of distinct fractions was obtained. It was also found that the molecular weight in the presence of urea or guanidinium chloride is less than 10,000.

A rather low molecular weight is also indicated by the observation of Bakay (145), who found that a large proportion of their histone was *dialysable through cellophane. The end-group analysis,* mentioned by Haley (94), is also reported in ref. 144. Bakay and Toennies have also confirmed the observation that DNA prepared from tumor tissues always contains an appreciable proportion of RNA (146).

Alfert and Geschwindt, in unpublished observations, have compared the ratio of histone to DNA (determined by means of the Feulgen and fast green, pH 8, reactions) in rat liver tumors induced by

particles as templates which either directly or indirectly guide the synthesis of peptide chains with amino acids in a definite order. No adequate scheme whereby this could occur has yet been proposed. The basic difficulty is that the number of distinct monomeric units available in the nucleic acids is much less than the number of amino acids in proteins, and it is difficult to see how a structure with only 4 (or occasionally 5) building blocks can guide the formation of structures with 20 or more.

To overcome this difficulty it has been suggested (138) that two or even three adjacent nucleotides are concerned in fixing the position of one nucleotide unit. This clearly provides a greater number of possible combinations; but no one has been able to offer a convincing picture of how such a scheme would work, so that it remains hypothetical (139). The finding of Gale (140) that the incorporation of radioactively labeled amino acids by disrupted *Staphylococcus* cells was stimulated by specific nucleotide groupings gave an exciting prospect of testing these ideas. However at the present time it is uncertain if the real stimulating agents are nucleotides, and further work must be awaited.

C. ROLE OF THE PROTEINS

Very little is known of the function of the proteins attached to DNA in different cells. The fact that the action of the transforming principles is brought about by purified DNA suggests that the proteins take no part in the conveyance of genetic information. They may by combination with phosphate and other exposed groups of the DNA prevent its combination with other proteins present in the nucleus. Other possibilities are that they may have functions in holding together the two strands of the DNA, that they may be part of the mechanism of duplication which operates in mitosis, or that they are part of the structural continuum that holds together the large number of DNA particles present.

It is found that the synthesis of DNA and histone occurs synchronously in mitosis. The residual protein of the chromosomes is more active metabolically than the histones (141) in amino acid incorporation experiments. It also appears to be present in greater quantities in metabolically active cells.

The significance of the fact that the basic protein is protamine in certain sperm cells and histone in most somatic cells is unknown.

13. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, *30*, 117 (1946).
14. Butler, J. A. V., Davison, P. F., and James, D. W. F., *Proc. Biochem. Soc.*, *54*, xxi (1953). *Cf.* Chargaff, E., in *Some Conjugated Proteins* Rutgers Univ. Press, 1953, p. 36
15 Schwander, H., and Signer, R., *Helv. Chim. Acta*, *52*, 853 (1949); *33*, 1521 (1950)
16. Kay, E. R. M., Simmons, N. S, and Dounce, A. L., *J. Am. Chem. Soc.*, *74*, 1724 (1952); Marko, A. M., and Butler, G. C., *J. Biol. Chem.*, *190*, 165 (1951).
17. Butler, J. A. V., James, D W. F., and Conway, B. E., *Trans. Faraday Soc.*, *50*, 1612 (1955).
18. Sevag, M. G., *Biochem. Z.*, *273*, 419 (1934); Sevag, M G., Lackman, D. B., ... J. M., Jordan,

19.   .   ,   ,
20. Jones, A. S., *Biochim. et Biophys. Acta*, *10*, 607 (1953).
21. Chargaff, E., and Zamenhof, S, *Nature*, *168*, 604 (1951).
22. Dutta, S. K., Jones, A. S., and Stacey, M, *Biochim. et Biophys. Acta*, *10*, 615 (1953).
23. Hunter, G. D., and Butler, J. A. V., *Biochim. et Biophys. Acta*, *20*, 405 (1956).
24. Westphal, O., and Bister, F., *Z. Naturforsch.*, *B7*, 148 (1952); Kirby, *Biochem. J.*, *64*, 405 (1956).
25. Kirby, K. S., *Biochem. J. (Proc.)*, *62*, 31 (1956).
26. Butler, J. A. V., Johns, E. W., Lucy, J. A., and Simson, P., *Brit. J. Cancer*, *10*, 202 (1956).
27. Crick, F. H. C., and Watson, J. D., *Proc. Roy. Soc., London*, *A223*, 80 (1954); *Nature*, *171*, 964 (1954).
28. Franklin, R. E., and Gosling, R. G., *Nature*, *172*, 156 (1953). Wilkins, M. H. F., Stokes, A. R., and Wilson, H. R., *ibid.*, *171*, 738 (1953).
29. Franklin, R. E., and Gosling, R. G., *Acta Cryst.*, *6*, 673, 678 (1953); *8*, 151 (1955).
30. Feughelman, M., *et al.*, *Nature*, *175*, 834 (1955).
31. *E. g.*, Butler, J. A. V., Johns, E. W., Lucy, J. A., and Simson, P., *Brit. J. Cancer*, *10*, 202, 1956
32. Dunn, D. B., and Smith, J. D, *Nature*, *174*, 305 (1954). Wacker, A, Trebst, A., Jackerts, D, and Weygood, F., *Naturforsch*, *96*, 616 (1954) Zamenhof, S., and Greboff, G., *Nature*, *174*, 306 (1954).
33 Wyatt, G R., and Cohen, S. S., *Biochem. J.*, *55*, 774 (1953); *Nature*, *170*, 1072 (1952).
34. Kahler, M., and Lloyd, B. J., *Biochim. et Biophys. Acta*, *10*, 353 (1953)
35 Ogston, A. G, and Cecil, R., *J. Chem. Soc., London*, *1948*, 1382. Ogston, A. G., and Fessler, J. H., *Trans. Faraday Soc.*, *47*, 667 (1951).
36. Shooter, K V., Pain, R. H, and Butler, J. A. V., *Biochim et Biophys Acta*, *20*, 497 (1956).
37.   .   ... *P   D   I   A Chem*
                                                            I.,
                                                           91
   Nature, *170*, 104, ...
   (1953).
38. Pouyet, J., *J. chim. phys*, *43*, 90 (1951); *Compt. rend.*, *234*, 152 (1952).
39. Conway, B. E., and Butler, J. A. V., *J. Polymer Sci.*, *12*, 199 (1954).
40. Butler, J. A. V., Conway, B E., and James, D. W. F, *Trans. Faraday Soc.*, *50*, 612 (1954).

butter yellow. In two tumors these ratios were indistinguishable from those obtained with normal control rats; in the third the ratio DNA/histone was increased. These results, therefore, do not support the claim of Stedman and Stedman (101) that the histone/DNA ratio is greater in tumors than in normal tissues.

An interesting hypothesis concerning the way in which information could be conveyed by the order of the bases in nucleic acids has been proposed by Crick, Griffiths, and Orgel (personal communication, based on paper to be published). The difficulty to be overcome is how to relate sequences of 2 or 3 nucleotides with particular amino acids. It is pointed out that while there are 64 ways of making up a group of 3 nucleotides from 4 such units, the number will be diminished if we exclude those which could be made by overlapping two adjacent sets. They find there are in fact twenty such combinations of three units which do not permit such overlapping.

Much evidence has been emerging, which is outside the scope of this review, that the formation of protein occurs from nucleotide-amino acid compounds. It would have to be demonstrated that each amino acid is associated with a distinctive trinucleotide. The decisive act of association would be effected in a specific enzyme for each amino acid. It does, in fact, appear that each amino acid has a distinct "activating" enzyme, but the nature of the combinations which they bring about is not well known

References

1. Davison, P. F , Conway, B. E., and Butler, J A. V , *Progress in Biophysics*, *4*, 148 (1954).
2 Allfrey, V. G., Mirsky, A. E., and Stern, H., in *Advances in Enzymology*, Vol. XVI, Interscience, New York-London, 1955, p. 411.
3 Chargaff, E., and Davidson, J. N., eds., *The Nucleic Acids*, Academic Press, New York, 1955.
4 Steiner, R. F., *Trans. Faraday Soc*, *48*, 1185 (1952)
5 Shooter, K. V., Davison, P. F, and Butler, J. A. V., *Biophys. et Biochim Acta*, *13*, 192 (1954)
6. Felix, K., Fischer, H., and Krekels, A., *Progress in Biophysics*, *6*, 1 (1956).
7. Dounce, A L., and Monty, K J, *J. Biophys Biochem Cytol*, *1*, 139, 155 (1955)
8 Davison, P F, and Butler, J A V, *Biochim et Biophys Acta*, *21*, 568 (1956)
9 Luck, J M, Kupke, D W, Rhein, A, and Hurd, M, *J Biol Chem*, *205*, 215 (1953)
10 Kupke, D W, Eldridge, N T, and Luck, J M, *J Biol Chem*, *210*, 295 (1954)
11. Welsh, R, *Biochim et Biophys Acta*, *16*, 171 (1955)
12 Fnck, G, *Biochim et Biophys Acta*, *19*, 352 (1956)

73. Cruft, H. J., Mauritzen, C. M., and Stedman, E., *Nature, 174*, 580 (1954).
74. Khouvine, Y., Gregoire, J., and Zalta, J. P., *Bull. soc. chim. biol., 35*, 244 (1953).
75. Ahlström, L., *Arkiv Kemi Mineral. Geol., A24*, No. 31 (1947).
76.

77.
78.
    (1954).
79. Crampton, C. F., Moore, S., and ·Stein, W. H., *J. Biol. Chem., 215*, 787 (1955).
80.
81.
82.   3).
83. Scanes, F. S., and Tozer, B. T., *Biochem., 65*, 565 (1956).
81. Crampton, C. F., unpublished experiments.
85.
86.
87.
87a. Shooter, K. V., and Davison, P. F., unpublished experiments.
88. Butler, J. A. V., Davison, P. F., James, D. W. F., and Shooter, K. V., *Biochim et Biophys. Acta, 15*, 224 (1954).
89. Cohen, S. S., *J. Biol. Chem., 158*, 255 (1945).
90 Gregoire, J., and Limozin, M., *Bull. soc. chim. biol., 36*, 15 (1954).
91. Smillie, L B., Marko, A. M., and Butler, G. C., *Can. J. Biochem. Physiol, 33*, 263 (1955).
91a. Oth, A , and Desreux, V., *Intern. Symp. Macromol. Chem , Israel, 1956; J. Polymer Sci., 23*, 713 (1957).
92. Stedman, E , and Stedman, E., *Cold Spring Harbor Symp. Quant. Biol., 12*, 224 (1947).
93. Bakay, B., Kolb, J. J , and Toennies, G., *Arch. Biochem. Biophys., 68*, 144 (1955).
94.   15 962 (1955).
95.
96.
97.   *Mineral. Geol., A26*, No. 20, 1 (1948).
98. Stedman, E., and Stedman, E., *Nature, 166*, 780 (1950).
99. Daly, M. M., Mirsky, A. E., and Ris, H., *J. Gen. Physiol , 54*, 439 (1950).
100. Crampton, C. F., Moore, S., and ·Stein, W. H., *Federation Proc., 14*, 198 (1955).
101   *Biol , 1*, 232 (1947).
102.
103.   ).
104. Kossel A., *The Protamines and Histones*, Longmans, Green, New York, 1928
105.
106.
107.   eds ,

108.   )53).
109.   3).
110.

41. Butler, J. A. V., and Conway, B. E., *Nature*, *172*, 153 (1953) Conway, B. E ; *J. Polymer Sci.*, *18*, 257 (1955). Eisenberg, H., and Pouyet, J., *J Polymer Sci.*, *13*, 85 (1954).

42. Jordan, D. O., Mathieson, A. R., and Matty, S., *J. Chem. Soc., London, 1956*, 154, 158

43 Cox, R. A., and Peacocke, A. R., *J. Chem Soc., London, 1956*, 2499.

44. Mathieson, A. R., and McLaren, J. W., *J Chem. Soc., London, 1956*, 303.

45. Zamenhof, S, Griboff, G., and Marullo, N, *Biochim et Biophys. Acta, 13*, 459 (1954).

46. Goldstein, G., and Stern, K G., *J. Polymer Sci*, *8*, 687 (1950). Thomas, R., *Experientia, 7*, 261 (1951); *Biochim. et Biophys. Acta, 14*, 231 (1954). Laland, S. G., Lee, W A, Overend, W. G., and Peacocke, A. R., *Biochim. et Biophys Acta, 14*, 386 (1954).

47. Doty, P., and Thomas, C. A., *J. Am Chem. Soc., 78*, 185 (1956).

48. Cox, R. A., and Peacocke, A. R, *J. Chem. Soc, London, 1956*, 2499.

49. Dekker, C. A., and Schachman, H. K, *Proc. Natl. Acad. Sci. U. S., 40*, 894 (1954).

50 Doty, P., and Rice, S. A., *Biochim. et Biophys. Acta, 16*, 446 (1955)   See Sadron, Ch., *3rd Intern. Congr. Biochem (Brussels), 1955, Report*, p. 134, also Doty, P., *ibid*, p. 135.

51. Shooter, K. V., Pain, R. H., and Butler, J. A. V., *Biochim. et Biophys Acta, 20*, 497 (1956)

52. Conway, B. E., and Butler, J. A. V., *J. Chem. Soc, London, 1952*, 3075.

53. Conway, B E, *J. Polymer Sci., 20*, 299 (1956).

54. Doty, P., *3rd Intern. Congr. Biochem (Brussels), 1955, Report*, p. 135.

55 Alexander, P., and Stacey, K. A., *Biochem. J., 60*, 194 (1955).

56 Alexander, P., and Stacey, K. A, *Nature, 175*, 500 (1955).

57 Shooter, K. V., and Butler, J. A. V., *Nature, 175*, 500 (1955); *Trans. Faraday Soc., 52*, 734 (1956).

58. Shooter, K. V., and Butler, J. A. V., *Nature, 177*, 1033 (1956)

59. Peacocke, R. A., and Schachmann, H K., *Biochim. et Biophys. Acta, 16*, 198 (1954)

60. Shooter, K. V., and Butler, J. A. V., *J. Polymer Sci., 23*, 705 (1957)

61. Shooter, K. V., private communication   H. K. Schachman, private communication.

62. Shooter, K. V., private communication.

63. Chargaff, E., Crampton, C. F., and Lipshitz, R., *Nature, 172*, 289 (1953); *J. Biol. Chem , 211*, 125 (1954). Lipshitz, R., and Chargaff, E., *Biochem. et Biophys Acta, 19*, 256 (1956).

64. ᵃ   ᵃ   (a, 16, 431 (1955).

65.   ᵃ   65, 133 (1956).

66.   ᵃ   ᵃ   ᵃ   ᵃ   ᵃ

67.   ᵃ   ᵃ   ᵃ   ᵃ   ᵃ

68 Bendich, A., Fresco, J. R., Rosenkranz, H. S., and Benzer, S. M., *J. Am. Chem. Soc., 77*, 3671 (1955).

69. Spitnik, P., Lipshitz, R., Chargaff, E, and Benzer, S. M., *J. Am. Chem Soc , 77*, 3761 (1955)

70 Hichens, M., Kent, P. W., and Ward, P. F. V., *Trans. Faraday Soc.*, in press.

71. Stedman, E., and Stedman, E., *Phil. Trans. Roy. Soc., B235*, 565 (1951)

72. Davison, P. F., James, D. W. F., Shooter, K. V., and Butler, J. A. V., *Biochim. et Biophys. Acta, 15*, 415 (1954).

The Preparation and Properties of Ribonucleic Acids from Rat Liver

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Early methods of preparing ribonucleic acid (RNA) from mammalian tissues and yeast involved the use of alkali (Levene & Bass, 1931), but it is now recognized that this process necessarily involves a considerable amount of degradation (cf. Allen, 1954). On the other hand, if very mild conditions are used there is a danger of degradation by ribonuclease during the preparation.

Attempts have been made to inactivate ribonuclease without affecting the RNA, but none of the substances used seems to be entirely satisfactory. Thus Grinnan & Mosher (1951) used 4M-guanidine hydrochloride as a denaturing agent, followed by heat to remove the protein. This yielded a product stated to have a sedimentation coefficient $S = 10$ s corresponding to a high molecular weight (about 260 000). Gulland, Barker & Jordan (1945) have shown, however, that guanidine hydrochloride denatures deoxyribonucleic acid, and the probability of such action upon RNA, followed by aggregation, cannot be overlooked. Kay & Dounce (1953) have used sodium dodecyl sulphate as a protein precipitant and they obtained material in 50 % yield having sedimentation coefficients of about 3s, corresponding to a molecular weight value of $2-3 \times 10^4$. It has since been shown by Allen (1954) that dodecyl sulphate does not completely inactivate ribonuclease under the conditions obtaining in this method of preparation. Strong salt will split a certain proportion of the cytoplasmic nucleoprotein, but only on prolonged standing, and

RNA thus obtained showed evidence of considerable enzymic depolymerization.

In the course of investigating these methods, it was found that after mild acid treatment a large part of the nucleic acid was easily separated from the associated protein and lipid by a short period of heating in salt solution. The protein flocculated and precipitated, leaving most of the nucleic acid in solution together with a small amount of protein which could readily be removed by digestion with chymotrypsin. It was also found that this process is effective with isolated cell fractions. The ribonucleic acids obtained have sedimentation coefficients in the range 2-3 s, corresponding to molecular weights of $2-3 \times 10^4$. It is possible that the ribonucleic acids of high molecular weight obtained by some other workers are artifacts produced by aggregation of particles as a result of the reagents employed. It must be admitted, however, that some depolymerization may occur during the heating process.

EXPERIMENTAL AND RESULTS

Preparation of ribonucleic acids from whole cytoplasm Freshly excised and chilled rat livers were minced and ⟨...⟩ Waring ⟨...⟩ lls were ⟨...⟩ erations ⟨...⟩ and as up to the heating process were carried out ⟨...⟩ as quickly as possible to minimize enzymic degradation The crude nucleoprotein was obtained as a white precipitate by

111. Dounce, A. L., *Exptl. Cell Research*, Suppl. 2, 103 (1952).
112. Dallam, R. D., and Thomas, L. E., *Biochim. et Biophys. Acta*, *11*, 79 (1953).
113. Vendrely, R., and Vendrely, C., *Nature*, *172*, 30 (1953). Vendrely, C.,
     Knobloch, A., and Vendrely, R., *Biochim. et Biophys. Acta*, *19*, 472 (1956).
114. Knobloch, A., and Vendrely, R , *Nature*, *178*, 261 (1956).
115. Mayer, D. T., and Gulick, A., *J Biol. Chem* , *146*, 433 (1942).
116 Stedman, E., and Stedman, E., *Nature*, *152*, 267 (1943).
117. Wang, T.-Y., *Microfilm Abstr.*, *11*, 813 (1951) Wang, T.-Y., Kirkham, W. R ,
     Dallam, R D., Mayer, D. T., and Thomas, L E , *Nature*, 165, 974 (1950).
     Wang, T.-Y., Mayer, D. T., and Thomas, L E , *Exptl Cell Research*, *4*, 244
     (1953).
118 Hamer, D., *Brit. J. Cancer*, *7*, 151 (1953).
119. Mirsky, A E., and Ris, H., *J. Gen. Physiol.*, *31*, 1, 7 (1947).
120 Dounce, A I   Sympos 110 119 (1949)
121.
122.
123.
124.   , J N.,

125. Dounce A I   and Mc Ann 77 T   .   11 1
126.
127.
128
129. Mirsky, A. E., and Ris, H , *Cold Spring Harbor Symp. Quant. Biol.*, *12*, 143
     (1947)
130. Crampton, C. F., Lipshitz, R , and Chargaff, E., *J. Biol Chem.*, *206*, 499
     (1954).
131. Watson, J. D., and Crick, F. H. C., *Nature*, 171, 964 (1953)
132. Schwartz, D., *J. Cellular Comp Physiol.*, *45*, Suppl 2, 171(1955). Platt, J.
     R., *ibid.*, *41*, 181 (1955).
133. Levinthal, C , *Proc. Natl. Acad. Sci. U. S.*, *42*, 436 (1956).
134. Butler, J. A. V., *Radiation Research*, *4*, 20 (1956).
135. Mazia, D., *Cellular Aspects of Basic Mechanisms in Radiobiology*, National
     Research Council, 450, Washington, 1956; *Henry Ford Hospital Sym-
     posium on Enzymes*, Academic Press, New York, 1956, p. 272
136. Levinthal C   Proc Natl Acad Sci U S   42 394 (1956)
137.
138.   (1955).
139.
140.   port, p. 345.

141.   iol., 38, 415
     (1955)
142. Alfert, M., *J. Biophys. Biochem Cytol.*, *2*, 109 (1956).
143.
144.   W., and

145. Bakay, B , in press.
146. Bakay, B., and Toennies, G , in press.

are given in Table 3 as moles of base:moles of adenine.

It is seen that the ribonucleic acids obtained from cell fractions by the heat preparation do not differ appreciably in composition from the whole cytoplasm RNA as regards base composition. These results agree closely with those of Crosbie, Smellie & Davidson, which are included in Table 3. The ratio of 6-amino base to 6-oxo bases is constant and near to unity for all the preparations, as found by Elson & Chargaff (1955). [That the values are uniformly less than one may be due to the method of hydrolysis (Crosbie et al. 1953)].

RNA prepared from fraction II by the detergent method differs markedly in base composition from RNA prepared by the heating method, as shown by the ratio (adenine+uracil):(guanine+cytosine) However, the purine:pyrimidine ratio is unaffected, as is the ratio of 6-amino groups:6-oxo groups.

Sedimentation analysis

The sedimentation behaviour of the preparations was studied with a Spinco analytical ultracentrifuge. As a rule, only one broad peak was observed, as is shown for a 1 % solution of RNA in 10 4 M-NaCl in Fig. 1 (c) ($S_{20, w} = 2.35$s). However, in a few cases, a double peak was observed; e.g. with RNA sample 48, obtained by heating the whole cytoplasm homogenate at 100° for 10 min., without subsequent treatment with chymotrypsin, a 1 % solution in water gave two peaks, as shown in Fig. 1 (a), with $S_{20, w} = 4.06$s (A) and 2.32s (B). It was thought that the material A might be an aggregated form of B and 2 M-urea was added to the solution in an attempt to break up such aggregates. Under these conditions, however, two peaks were again observed [Fig. 1 (b)] with sedimentation coefficients, corrected for changes of density and viscosity ($S_{20, w} = 2.9$s and 2.1s). After dialysing

out the urea the sedimentation coefficients returned approximately to the original values ($S_{20, w} = 4.23$s and 1.91s). These results show that A undergoes some changes of shape or aggregation in urea, but it does not become identical with B. This means that they are probably two distinctly different substances. Since the N/P ratio for this preparation was 2 05, it contains about 20 % of protein and the component A is thus probably a nucleic acid–protein complex. It may be noted that in preparations treated with chymotrypsin to remove protein no such double peaks have been observed.

An estimate of the molecular weight, as calculated from the mean sedimentation coefficients (determined in 1 % solution in 0·14 M-NaCl) and the diffusion coefficients, determined in the Perkin-Elmer electrophoresis apparatus (1 % solutions of RNA, which had been dialysed against phosphate buffers, pH 6·8, at ionic strength 0·2) is given in Table 4. The value of the partial specific volume of RNA used in the calculations was 0 523 (Delcambe & Desreux, 1950).

Such values, calculated from sedimentation and diffusion coefficients measured at finite concentration, can be only approximate. However, since Grinnan & Mosher (1951) found that S_{20} for their RNA of rat liver varied little with concentration up

only 20 % between zero concentration

is likely that these values for the molecular weights are of the right order of magnitude

Table 4. Sedimentation and diffusion data for RNA from cell fractions (see text)

Fraction	$S_{20, w}$(s)	$10^7 D_{20, w}$	Mol.wt.
II (microsomal)	3 70	7·0	26 000
III (supernatant)	2 4	10 6	11 000

(a)   (b)   (c)
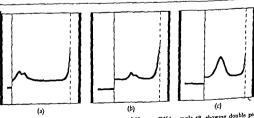
Fig.   double peak in water
                                          action III, showing

acidifying to pH 4·5 with м-HCl and was collected by centrifuging at 1200 g for 15 min. This step was found to be essential for efficient removal of protein at later stages. ~~...........~~

lipid material.

A fairly complete separation of the protein and nucleic acid was obtained by heating the acid precipitate at neutrality in salt solution. After dispersing in 2м-NaCl (11/150 g. of rat liver) and adjusting to pH 7 with 0·1 м-NaOH, the suspension was heated for 10 min. at 80° with continuous mechanical stirring. These optimum conditions were found from trial runs, shown in Table 1, yields being calculated as a percentage of the RNA present in the starting material as given by Thomson, Heagy, Hutchison & Davidson (1953). While high temperatures gave products with high sedimentation coefficients, these were not the result of increased extraction of highly polymerized material, as the yields were usually lower. Northrop & Sinsheimer (1954) have found aggregation of RNA to occur on heating at high temperature for several minutes.

After heating and cooling rapidly again to room temperature, the flocculated precipitate was removed by centrifuging, leaving a lemon-yellow supernatant. This contained the extracted RNA, which could be precipitated by the addition of 2 vol. of 98% ethanol to give products with N/P ratios in the region of 2. Purified products were obtained by digesting the supernatant with chymotrypsin at pH 7·5 for 16 hr. before precipitating with ethanol. The RNA was then reprecipitated from isotonic NaCl solution and finally washed with 70, 75, 80 and 90% (v/v) ethanol-water mixtures. It could be dried with ethanol and acetone, or by freeze-drying. This gave samples of RNA having N/P ratios of 1·7, which on hydrolysis and paper chromatography were shown to contain ribose, adenine, guanine, uracil and cytosine, with only a trace of thymine corresponding to less than 2% of DNA. The biuret reaction was negative and chromatography of the acid hydrolysate revealed less than 1% of protein.

Preparation of ribonucleic acid from cytoplasmic fractions

Three cell fractions were prepared by the method of Schneider & Hogeboom (1951). The cytoplasmic homogenate was prepared in 9 vol. of 0·25м-sucrose with a Potter-Elvehjem homogenizer fitted with a Teflon (polytetrafluoroethylene) pestle. Nuclei, unbroken cells and cell debris were removed by centrifuging at 1200 g for 15 min.

Fraction I was brought down at 10 000 g for 20 min and washed once by resuspending in 0·25м-sucrose and centrifuging again. Fraction II was obtained at 36 000 g for 1·5 hr, leaving the supernatant (fraction III).

Fractions I and II were suspended in 0·14м-NaCl and all three were acidified and heated, as in the whole cytoplasm preparation. Fraction I, predominantly a 'mitochondrial' fraction, was contaminated with considerable amounts of 'microsomal' RNA (see Table 2), owing to the inadequate fractionation obtained by the method of Schneider & Hogeboom. The degree of contamination depends on whether the 'fluffy' layer (Schneider & Hogeboom, 1951) is added to fraction I (preparation A) or fraction II (preparation B). Sufficient resolution is obtained between fractions II and III to justify a comparison of their properties.

RNA was also prepared from fraction II by the sodium dodecyl sulphate method of Kay & Dounce (1953).

Base analysis of ribonucleic acids

Base analysis of the various preparations was made by the method of Wyatt (1951). Hydrolysis was carried out with 72% HClO₄ for 1 hr. Results

Table 1. Influence of experimental conditions on amount and nature of the product

Conditions of heating in 2м-NaCl	Product		Yield (pptd by ethanol) (%)
	N/P	$S_{20,w}(s)$	
50°/30 min	No flocculation		—
60°/10 min.	1·93	2·18	20
80°/10 min.	1·70	2·12	50
80°/30 min.	1·71	3·38	20
100°/5 min.	2·01	5·2	20
100°/10 min.	1·87	2·76	15
100°/30 min.	—	—	Negligible

Table 2. Yields of RNA prepared from cell fractions

	Fraction no. (mg /250 g of liver)		
Preparation	I	II	III
A	340	80	225
B	100	160	215

Table 3. Base composition of RNA prepared from rat liver

SDS, Sodium dodecyl sulphate as protein precipitant. (Molar ratios with respect to adenine = 1.)

Preparation	Guanine	Cytosine	Uracil	6-Amino:6-keto	Purine: pyrimidine	(Adenine + uracil):(guanine + cytosine)
RNA sample 48 Whole cytoplasm	1·74	1·53	0·82	0·99	1·17	0·56
RNA sample 55 Whole cytoplasm	1·76	1·51	0·83	0·97	1·18	0·60
RNA sample 101/1 Fraction II	2·09	1·75	0·91	0·92	1·16	0·50
RNA sample 101/3 Fraction III	1·80	1·56	0·82	0·98	1·18	0·54
RNA Whole cytoplasm (SDS)	2·75	2·23	0·73	0·93	1·27	0·35
RNA sample 101/4 Fraction II (SDS)	2·47	2·01	0·90	0·89	1·19	0·42
RNA sample 100/2 Fraction II (SDS)	2·58	2·13	0·83	0·92	1·21	0·39
RNA Whole cytoplasm*	1·75	1·36	0·81	0·93	1·27	0·58

* Crosbie, Smellie & Davidson (1953): HClO₄ hydrolysis.

A New Method for the Isolation of Deoxyribonucleic Acids: Evidence on the Nature of Bonds between Deoxyribonucleic Acid and Protein

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When homogenized mammalian tissue was extracted with a two-phase phenol–water mixture at room temperature ribonucleic acids (RNA) were released into the aqueous layer, whereas deoxyribonucleic acids (DNA) remained completely insoluble with the denatured protein. This method has been developed as a preparative procedure for RNA (Kirby, 1956b).

Replacement of the water by solutions of certain anions brought DNA as well as RNA into the aqueous layer and the effectiveness of some of the salts used has been reported in a preliminary note (Kirby, 1956a).

Most of the tests have been carried out with rat liver, but similar results have been obtained with rat kidney, spleen, testis, a hepatoma and calf thymus glands. The tissue was homogenized with isotonic solutions of the salts, adjusted to pH 6·5 in order to avoid complications by release of nuclear materials through osmotic or pH effects. DNA was liberated when potassium cyanide or ethylenediamine was used at pH 10, but remained insoluble if the pH was 6·5.

EXPERIMENTAL

Test for the release of deoxyribonucleic acids

The salt solutions were prepared either directly from the sodium salt or by weighing the acid, titrating it with N-NaOH to pH 6·5 and adjusting to the correct volume with water. When the sodium salt was insoluble at 0·15 M-concentration, as with riboflavin phosphate, nitroso-2-naphthol-3·6-disulphonate (nitroso R salt) and spermine phosphate, a saturated solution was used. Complexing agents which were insoluble in water were dissolved in 1 or 2 ml of 2-methoxyethanol, then water was added and the mixture used with any precipitate present.

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Pieces of tissue (1·5 g.) were treated with the solution of the compound to be tested (20 ml.) in an all-glass tissue grinder and the mixture was poured off from any fibrous matter which remained at the bottom of the tube. Phenol [(90 %, w/w); 20 ml.] was added to the tissue mixture and the two-phase system was stirred or shaken vigorously for 1 hr. at room temperature. The extract of phenol and water was then centrifuged at 1000 g for from 10 min. to 1 hr., depending upon the viscosity of the aqueous layer. A volume (1 ml.) of this aqueous supernatant layer was withdrawn. Dische's (1930) reagent (2 ml.) was added and the mixture was heated on a boiling-water bath. The development of a blue colour was a positive indication of DNA. Another 1 ml. portion of the aqueous layer was withdrawn, 1 ml of ethanol added and the mixture shaken vigorously. A fibrous precipitate showed that DNA was present. The precipitation test was essential where the Dische colour was inhibited by the salt in solution, as for example with KSCN, Na₂S₂O₃ and p-aminosalicylate. The Dische test was then applied to the precipitate after washing and dissolution.

It was essential to homogenize the tissue in the salt solution first and then to treat with the phenol, since no DNA was released by treating tissues with water and phenol and subsequently adding the effective salts.

Extraction of liver homogenates with n-butanol and protein denaturants

Rat liver (7·5 g.) was homogenized in water (100 ml.), 20 ml. portions were taken and the various compounds shown in Table 1 added, and each was then shaken with n-butanol (20 ml.). When the liver was homogenized in 0·15 M-sodium salicylate and treated with the same additives as above, exactly the same results were obtained.

Rat liver (1·5 g.) was homogenized in 20 ml. of 0·15 M-sodium salicylate; resorcinol (10 g.) was added and the mixture shaken, but no DNA could be detected after removal of insoluble material by centrifuging. Addition of n-butanol (8 ml.) and n-hexylresorcinol (6 g.) with the same homogenate and subsequent separation resulted in a positive test for DNA being given by the aqueous layer.

Fig. 2. Titration curves of RNA sample 55 (from whole cytoplasm): I, starting from neutrality to pH 2·6 and then to pH 11, II, starting at pH 7 to pH 11 and then to pH 2·6.

Titration curves

The preparation RNA sample 55 was titrated from neutrality both in the acid and alkaline direction and the pH values were obtained with a Doran Alkacid glass electrode (L.S B. Components Ltd., Stroud), and in each the forward titration was succeeded by a back titration through the whole accessible range of pH (Fig 2). No significant amount of hysteresis is observed between the forward and back titrations.

DISCUSSION

The method of preparation of RNA by heat denaturation of the protein has the great advantage of speed, since the time during which the nucleoprotein preparation is above 3° is much less than with some other processes It would appear that as the RNA is quite stable after the heating process, the ribonuclease present is removed with the flocculated protein The molecular weight of the product is similar to that of the preparation of Kay & Dounce (1953) and Volkin & Carter (1951). The higher molecular weights found by Grinnan & Mosher (1951) are not obtained. It is not certain, however, that no degradation occurs during the preparation, especially since heating for a longer period produces a more degraded product The absence of hysteresis in the titration curves means that in the preparation examined no evidence of a hydrogen-bonded structure was obtained

SUMMARY

1. A method of preparation of ribonucleic acid from rat liver is described involving heat denaturation and separation of the protein and lipid components. It has also been applied to cytoplasmic fractions. The yield is, in general, not greater than 50 %.

2. The ribonucleic acids obtained have molecular weights between 10 000 and 30 000. The composition of the ribonucleic acid obtained from the microsome fraction differs appreciably from that obtained by the sodium dodecyl sulphate method.

REFERENCES

Allen, F W (1954) Annu. Rev. Biochem 23, 99
Cohen, S. S. & Stanley, W. M (1942). J. biol Chem. 144, 589
Crosbie, G W., Smellie, R. M S & Davidson, J. N (1953). Biochem J. 54, 287
Delcambe, L. & Desreux, V. (1950). Bull Soc. chim Belg 59, 521.
Elson, D & Chargaff, E. (1955). Biochim. biophys Acta, 17, 367.
Grinnan, E. L. & Mosher, W. A. (1951) J biol. Chem. 191, 719
Gulland, J. M., Barker, G. R. & Jordan, D O. (1945) Annu. Rev. Biochem. 14, 175
Kay, E R M & Dounce, A. L. (1953). J. Amer. chem. Soc. 75, 4041
Kerr, S E. & Seraidarian, K (1949). J. biol Chem. 180, 1203
Levene, P A & Bass, L W (1931) Nucleic Acids. New York: Chemical Catalogue Co.
Northrop, T. G & Sinsheimer, R L. (1954). J. chem. Phys. 22, 703
Schneider, W C. & Hogeboom, G H. (1951). Cancer Res 11, 1.
Thomson, R. Y , Heagy, F. C , Hutchison, W C. & Davidson, J. N (1953) Biochem. J 53, 460.
Volkin, E. & Carter, C E. (1951). J. Amer. chem. Soc. 73, 1516.
Wyatt, G. R. (1951) Biochem J. 48, 581, 584.

dichlorobenzene was raised to boiling point (176°) during about 20 min. and heating was continued for another 40 min. The contents of the tube were by then a pale yellow, but no insoluble matter had separated. The tube was opened carefully, after freezing in solid CO_2, and the contents were quantitatively transferred to a small flask (B10 standard ground glass joint) which was attached to a rotary evaporator. . . . .

The residue . . . . this solution . . . .
matography (Whatman paper no. 4). The solvents used were methanol–11·6N-HCl–water (7:2:1) (Kirby, 1955). After development (16 hr.) the paper was dried at room temperature, the spots were marked with the aid of a Hanovia Detectolite lamp (Hanovia Ltd., Slough, Bucks) and the bases eluted with 5 ml of solvent. To elute thymine, cytosine and adenine 0·1N-HCl was used, and 0·5N-HCl for guanine. The estimations were carried out in the usual way

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reducing agent of Fiske & Subbarow (1925).

Estimation of amino acids

DNA (15 mg.) was weighed into a tube and mixed with 5·8N-HCl (0·5 ml). The tube was sealed and the mixture allowed to stand until the . . . .

. . . .
transferred to a small flask (B10 standard ground glass neck) which was attached to a rotary evaporator and the HCl removed at 40°. Water (0·5 ml.) was added several times and the HCl was left. A volume (0·1 ml.) of water–acetic acid (4:1, v/v) was added, and when as much as possible of the residue had been dissolved it was transferred to a small tube and centrifuged. A portion (0·01 ml.) of this solution was used for the determination of P.

Paper chromatography was carried out with Whatman no. 1 paper. A sheet 47 cm. × 16·5 cm. was used for single-way chromatography and one 47 cm. × 35 cm. for chromatography and electrophoresis. A volume (0·02 ml.) of the hydrolysis solution was applied to each paper and both were chromatographed by upward development along the longer length for 20 hr. A mixture of ethyl methyl ketone–acetic acid–water (3:1:1, by vol.) was added as a developing solvent, which rose about 40 cm. in the development time. The papers were dried at room temperature. The smaller paper was sprayed with ninhydrin solution, and the colour was allowed to develop during 48 hr., after which the paper was sprayed with a solution of copper acetate (100 mg.) in water (6 ml) and ethanol (94 ml.), and the spots corresponding to lysine (R_F 0·11) and arginine (R_F 0·16) were removed for quantitative estimation.

The larger paper was prepared for electrophoresis as follows: molten paraffin wax was applied to a depth of about 8 mm. along the shorter lengths of the paper; holes were punched at the centres of the waxed edges and, after spraying the paper evenly with 0·033M-acetic acid, it was hung by two hooks in a Perspex electrophoresis cabinet with 0·033M-acetic acid as the conducting solution. Electrophoresis (0·8 mA. at about 260 V. constant current) was allowed to proceed for 15 hr., when the paper was removed, dried at room temperature and sprayed with ninhydrin

solution and the colours were developed in the dark for 48 hr.

The ninhydrin colours were converted into their copper complexes. The pink spots were cut out, the colour was eluted with 80% (w/w) methanol–water (3 ml) and the absorptions were read at 500 mμ on a Unicam spectrophotometer.

Standard solutions of the amino acids (0·2 or 0·1%) were chromatographed in . . . . and . . . . of t . . . . val . . . . con . . . . mg. of amino acid in 5 ml of

Test for the presence of ribonucleotides in deoxyribonucleic acid preparations

DNA preparation (7·5 mg.) was heated in a sealed tube with N-HCl (0·05 ml.) for 1 hr. at 100°. A portion (0·01 ml.) of this solution was chromatographed on Whatman no. 4 paper with the solvent system ethyl methyl ketone–acetic acid–water (3:1:1, by vol.).

Ribose, if present, was detected by spraying the paper with aniline hydrogen phthalate (Partridge, 1949) and heating at 100° for 5 min.

Examination of the developed, dried paper with a Hanovia Detectolite lamp showed a brilliantly blue fluorescent spot (R_F 0·51) in addition to a number of other absorbing spots. A blue fluorescent spot with similar paper-chromatographic and ultraviolet light-absorbing characteristics was produced by heating guanine (1 mole) with deoxyribose (5 moles) with N-HCl at 100° for 1 hr. This blue fluorescent spot had the same R_F in the above solvent and in a non-acidic one (ethyl methyl ketone–tert.-butanol–water 60:25:15) (R_F 0·4) as that produced by heating DNA from rat liver, calf thymus or herring sperm with N-HCl. The nature of the compound is unknown, but adenine, cytosine and thymine when heated with deoxyribose in N-HCl at 100° produced no fluorescent material. The blue fluorescence was quenched by ammonia, but the substance was eluted with N-HCl, and had λ_{max} 259 mμ, λ_{min} 240 mμ, in alkali λ_{max} was 235 mμ and an inflexion appeared at 260 mμ.

This fluorescent component is possibly the same as that described by Dunn (1955) and by Levy & Snellbaker (1953). These authors reported a white fluorescent spot which appeared on paper chromatography of a hydrolysate of calf thymus DNA by N-HCl. Dunn was of the opinion that the substance was a guanine derivative but reported that guanine deoxyriboside did not produce a fluorescent component with N-HCl unless phosphoric acid was present.

RESULTS

The compounds that have been tested with rat liver are shown in Table 2.

Absorption of DNA by collagen. The ability of some bivalent metal ions to promote the absorption of DNA by an insoluble protein was studied by measuring the uptake of DNA by collagen which had been treated with various metal ions. The results are shown in Table 3.

Table 1. *Tests for release of deoxyribonucleic acids with different protein denaturants*

Rat liver (7·5 g.) was homogenized in 100 ml. of water. Portions (20 ml) were treated with the additives shown and then shaken with 20 ml. of *n*-butanol.

Additive	Dische reaction	Addition of ethanol
None	+ –	–
Sodium dodecyl sulphate (1 g.)	+ + +	Fibrous
Cetyltrimethylammonium chloride (1 g)	–	–
Urea (4·8 g)	–	–
Guanidine hydrochloride (7 65 g.)	+ –	–

Absorption of deoxyribonucleic acid by collagen

Collagen (standard hide powder) was extracted with cold water in a Soxhlet apparatus overnight and then well washed with water, most of which was removed by suction at the pump.

For treating the collagen 0 1 M-solutions of potassium citrate ($K_3C_6H_5O_7,H_2O$), sodium acetate ($NaOAc,H_2O$),

citrate (8 8 g.) were dissolved in water, N-NaOH (26 ml.) was added and the solution was made up to 500 ml Ferrous sulphate (13 9 g) was dissolved in 250 ml. of 0 1 M-potassium citrate; N-NaOH (10 ml.) was added and the mixture was made up to 500 ml.

The wet hide powder (4 g.) was mixed with 100 ml. of each of the above salt solutions and the mixtures were shaken occasionally during 1 hr., after which the solutions were filtered off. The metal-collagen complexes were suspended in water (100 ml.) and filtered off four times during th

treated collagens were weighed out, and to the first was added water (10 ml.), to the second rat-liver DNA solution (10 ml) and to the third calf-thymus DNA solution (10 ml) were added, and the mixtures were allowed to remain in a cold room. The light absorption at 2

Isolation of deoxyribonucleic acids from rat liver

It was advantageous to allow the rats to fast for 24 hr. before removing the livers The animals were killed by breaking their necks, the livers were removed and immediately dropped on to solid CO_2. The pooled rat livers (75 g) were broken down in a high-speed mixer (45 sec.) with a solution of sodium *p*-aminosalicylate (600 ml. of 6% w/v; not necessarily all added at once). The mixture was poured through a Büchner funnel to remove fibres and debris, and the filtrate was stirred while 600 ml. of 90% (w/w) phenol was added quickly. Stirring was cont

removed by suction. The phenolic layer and the insoluble material were washed once with a small quantity of 6% (w/v) sodium *p*-aminosalicylate solution, and the aqueous layer was separated by centrifuging. The combined aqueous layers (400 ml.) were stirred and 2-ethoxyethanol (400 ml.) was added. The fibrous precipitate was removed with a glass rod and placed in water (100 ml.) immediately. The DNA dissolved after shaking for 30 min and sodium *p*-aminosalicylate (6 g.) was added and the DNA was precipitated again with 2-ethoxyethanol (100 ml.). In each case a flocculent precipitate of RNA remained in the water-ethoxyethanol mixture. The DNA precipitate dissolved quickly in water (100 ml.); sodium acetate ($NaOAc,H_2O$, 4g.) was added and the DNA precipitated with 2-ethoxyethanol (100 ml.). This precipitate was dissolved in 50 ml. of water, sodium acetate (2 g.) and ribonuclease (1·5 mg. in 1 ml of water) were added and the mixture was allowed to remain at 2° for 16 hr. The DNA was precipitated by 2-ethoxyethanol (50 ml) and as much as possible of the solvent removed from the precipitate before it was dissolved in water (33 ml). When dissolution was complete

layer was run on The top layer was separated from any insoluble material and centrifuged in polyethylene centrifuge tubes in a Servall centrifuge at 10000 g for 1 hr. The clear organic layer was carefully poured from any insoluble sediment, a few drops of toluene were added and the mixture was dialysed twice against water (2 l. each time), and twice against 1% sodium acetate (2 l. each time). The contents of the bag were then removed, centrifuged, made up to 4% (w/v) with respect to sodium acetate (the volume is usually about 100 ml.), and the DNA was precipitated with an equal volume of 2-ethoxyethanol. The fibrous precipitate

liver, which indicated that there were few losses, as Thomson, Heagy, Hutchison & Davidson (1953) reported values of 21·0-29·7 mg of DNA P/100 g. of rat liver.

DNA has also been prepared in exactly the same way except that the livers were broken down in sodium trichloroacetate (0·3 M) or sodium benzoate (0·3 M). The yields were about the same.

DNA which had been precipitated each time with ethanol instead of 2-ethoxyethanol could be separated from ribonucleotides present by dissolution in water and precipitating twice with 2-ethoxyethanol in the presence of sodium acetate.

Determination of base ratios

The base ratios of the DNA were determined by hydrolysis with formic acid in the following manner (Vischer & Chargaff, 1948; Wyatt, 1951): DNA (2·5 mg) was weighed into a test tube made of heavy walled Pyrex glass (9 mm. × 70 mm.); the neck was constricted and formic acid (Analar; 0·5 ml. of 90%) was added. The tube was sealed at the constriction and allowed to remain until the DNA had dissolved. The tube was then placed in a large test tube containing 1 2-dichlorobenzene and fitted with a reflux condenser. The

Table 3. *Absorption of deoxyribonucleic acids by collagen*

	Rat-liver DNA						Calf thymus DNA					
	Original optical density (258 mμ)	Optical density after				DNA left in soln. (% after 14 days)	Original optical density (258 mμ)	Optical density after				DNA left in soln. (% after 14 days)
Cation		2 days	4 days	7 days	14 days			2 days	4 days	7 days	14 days	
Cu²⁺	0·725	0·54	0·495	0·435	0·37	49·9	0·655	0·545	0·455	0·38	0·31	47·5
Co²⁺	0·725	0·62	0·565	0·535	0·50	67·8	0·655	0·525	0·485	0·45	0·41	62·5
Fe³⁺	0·725	0·67	0·610	0·590	0·50	72·3	0·655	0·585	0·55	0·47	0·42	64·9
Mn²⁺	0·725	0·61	0·595	0·505	0·43	58·2	0·655	0·535	0·48	0·44	0·39	59·5
Zn²⁺	0·725	0·60	0·555	0·530	0·47	64·5	0·655	0·51	0·46	0·43	0·38	58·5
Na⁺	0·405	—	—	0·417	0·41	100	0·53	—	—	0·517	0·50	93·5
K⁺	0·405	—	—	0·405	0·405	100	0·53	—	—	0·525	0·53	100·0

Table 4. *Analyses and base ratios of deoxyribonucleic acids extracted in different ways*

Preparations were made with: (column *a*), 0·3M-trichloroacetate; (column *b*), 0·3M-p-aminosalicylate, (column *c*), 0·3M-benzoate. Base ratios are expressed as moles/100 moles of total bases.

		a	*b*	*c*
Guanine		21·1	21·3	22·1
Adenine		29·3	29·1	29·5
Cytosine	(base ratio)	20·2	20·1	19·8
Thymine		29·6	29·5	28·8
N (%)		13·20	11·9	12·5
P (%)		8·02	7·24	7·22
N/P		1·64	1·64	1·73
$\epsilon_{(P)}$ (258 mμ) (in 0·1 N-NaCl)		5900	6340	6580
$\epsilon_{(P)}$ (258 mμ) (in water)		7700	8150	8000
P (atoms/100 moles of total bases)		—	97	—

After the separation, DNA, RNA and small amounts of polysaccharides were present in the aqueous layer. Addition of ribonuclease brought about the breakdown of RNA, and DNA was separated from polysaccharides by extraction with 2-methoxyethanol from potassium phosphate solutions, in exactly the same manner as described earlier for RNA (Kirby, 1956b) To judge from the Dische reaction DNA was entirely extracted into the organic layer, which was dialysed to remove particles of small molecular weight, and the DNA was finally precipitated by ethanol. The product was shown by hydrolysis to contain amino acids and ribonucleotides, and DNA prepared by the sodium benzoate method had the highest content of amino acids Treatment with sodium dodecyl sulphate made little difference to the amino acid content, although there was some loss of material by this procedure. The amino acid content could be considerably or almost completely removed by increasing the concentration of the salts used for homogenizing from 0·15M to 0·3M. After this treatment DNA prepared with the aid of sodium benzoate contained approximately 1% of amino acids The use of sodium trichloroacetate reduced this content somewhat, and DNA prepared with sodium p-aminosalicylate contained only the faintest traces of amino acids. One difficulty arose in that, although it was not possible to detect ribonucleotides by the usual colour reaction,

hydrolysis of the DNA with N-HCl at 100° for 1 hr. followed by paper chromatography of the solution showed the presence of ribose. Obviously all the ribonucleotides had not been separated by dialysis despite the high ionic content. Complete separation of the fragments containing ribose was achieved by precipitating DNA with 2-ethoxyethanol instead of with ethanol.

Analyses of DNA. The base ratios and other analytical figures are shown in Table 4.

The $\epsilon_{(P)}$ (Chargaff, 1955) value is an indication that DNA was not degraded by treatment with phenol. The sodium benzoate preparation had a slightly higher guanine content than the DNA prepared with the aid of the other salts.

The DNA prepared by the three methods yielded varying amounts of amino acids upon hydrolysis, and it is probable that these originated from a protein or polypeptide firmly bound to the DNA. The amino acids have been estimated quantitatively to discover if their ratios bore any relationship to those found in histones as normally extracted from cell nuclei. Because of the large amount of glycine produced by decomposition of adenine and guanine under the hydrolytic conditions, it was essential to have a system that gave a good separation of this amino acid from those to be estimated. Paper chromatography with ethyl methyl ketone–acetic acid–water, followed by paper electrophoresis in 0·033M-acetic acid at right angles to the direction of

Since DNA was so easily liberated from tissues by the method described a number of preparations have been made from rat liver. The cell nuclei were not separated before the extraction. Sodium trichloroacetate, sodium p-aminosalicylate and sodium benzoate were chosen as salts for the preparation of homogenates since the first compound was a protein precipitant, the second had metal-complexing properties, whereas the third was not

known to possess either of these properties but produced a very viscous aqueous layer. Sodium p-aminosalicylate was used rather than salicylate since, after centrifuging, the aqueous layer was much clearer and easier to separate from the inter-facial insoluble material. This separation proved much more difficult with the mixture produced from the treatment with sodium benzoate because of the high viscosity of the aqueous layer

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Table 2. *Compounds tested for release of deoxyribonucleic acids*

Rat liver was homogenized with 0 15 M-solutions of the above compounds and the homogenate shaken with an equal volume of 90 % phenol. After centrifuging, the aqueous layer was tested for DNA. A strong Dische colour was produced by treatment with the effective compounds; a weaker colour by moderately effective compounds and in these the precipitate was less fibrous and the amount variable.

Anions which were effective	Anions which were moderately effective	Compounds which released no DNA
m-Aminobenzoate	0 66 M-Chloride	Acetate
2-Amino-1-naphthylsulphate	0·3 M-Chloride + o-phenanthroline	Acetylacetone
p-Aminosalicylate	Decanoate	Adrenaline
Aurine tricarboxylate	Diphenylacetate	Ascorbate
Azide	2·Ethylbutyrate	0 3 M-Chloride
Benzoate	Gentisate	Cholate
p-tert.-Butylbenzoate	Hexahydrobenzoate	Citrate
Cupferron	m-Hydroxybenzoate	Cyanide
1.2-cycloHexyldiamine-NNN'N'-	Naphthalene-2-sulphonate	Cysteine
tetracarboxylate	1-Naphthol-4-sulphonate	3 4-Dihydroxy benzoate
Diethyldithiocarbamate	1-Naphthylamino-4-sulphonate	1·8-Dihydroxynaphthalene-3 6-
Diphenate	isoNicotinate	disulphonate
2 6-Dipicolinate	Perfluoro-octanoate	Dihydroxyphenylalanine
Flavianate	Sulphosalicylate	1:2-Dimercaptopropanol
Fluoride		3 4-Dimercaptotoluene
3-Hydroxyanthranilate		NN-Dimethylglycine
Kynurenate		αα'-Dipyridyl
Methylene disalicylate		Dithio-oxamide
Methyl orange		Ethyl acetoacetate
Nicotinate		Ethylenediamine
2-Nitroso-1-naphthol-4-sulphonate		Ethylenediamine-NNN'N'-tetra-acetate
Orange II		Glycerophosphate
N-Phenylanthranilate		Glycine
2-Phenylphenanthrene-3 2'-		Hexametaphosphate
dicarboxylate		Histidine
Phenylphosphate		p-Hydroxybenzoate
Phthalate		8-Hydroxyquinoline
Picolinate		8-Hydroxyquinoline-5-sulphonate
Quinaldinate		Kynurenine
Quinolinate		Methylmalonate
Salicylate		2-Naphthylamine-4 8 disulphonate
Sorbate		1-Nitroso-2-naphthol-
Thiocyanate		3 6-disulphonate
Trichloroacetate		4-Nitrosoresorcinol
Xanthurenate		Orotate
		Oxalate
		Perchlorate
		o-Phenanthroline
		Phosphate
		Proline
		Pyridoxine
		Pyrophosphate
		Riboflavin phosphate
		Salicylaldoxime
		Spermine phosphate
		Tartrate
		Thioacetamide
		Thioglycollate
		Tryptophan

DISCUSSION

The separation of deoxyribonucleic acids from nucleoproteins has been the subject of several procedures, and these have been adequately discussed previously (Chargaff, 1955; Frick, 1954a, b; Jones & Marsh, 1954). A characteristic feature of the problem has been the varying degree of difficulty with which the DNA is separated from the protein in different species. Herring-sperm DNA was split quite easily from the protamine by treatment with м-NaCl. This method is not generally applicable since, for example, the nucleoprotein of *Mycobacterium phlei* was unaffected by saturated NaCl (Jones & Marsh, 1954). The application of sodium dodecyl sulphate to the separation of DNA (Marko & Butler, 1951) was a considerable advance, but here again it was much more difficult to obtain protein-free DNA from bull testis than from calf thymus gland with this method.

The present study has shown that a considerable number of salts may be used in conjunction with phenol to liberate DNA from mammalian tissues, but the salts were not equally efficacious in removing protein.

Phenol was as essential to the reaction as the salt in solution and no DNA was released when phenol was replaced by resorcinol or urea (4м), and very little when *n*-butanol or guanidine hydrochloride (4м) was used in conjunction with sodium salicylate solution. Sodium dodecyl sulphate produced a very viscous solution of DNA but cetyltrimethyl-ammonium chloride produced none at all.

The function of the phenol is probably that of a protein solvent, in that it extracts protein which has been separated by the salt in solution. Denaturation of the proteins may be incidental to the reaction, since the more usual protein denaturants urea and guanidine had no effect. The considerable power which phenol possesses to extract proteins from aqueous solution has been demonstrated by Grassmann & Deffner (1953).

To some extent the activity of the salts is related to their ability to interact with proteins. Trichloroacetate, toluenesulphonate, fluoride and thiocyanate ions are much more strongly bound by albumin than are chloride and perchlorate (Scatchard & Black, 1949), and this difference in binding corresponds to the ability of the anions to release DNA. Naphthalene-2-sulphonate showed very little activity in the DNA test, although this anion is normally very strongly bound by proteins (Steinhardt, 1941). 1-Naphthol-4-sulphonate was not much better but 2-nitroso-1-naphthol-4-sulphonate was very much more effective, and the increased activity may be related to the presence of a metal-complexing group. Probably most of the compounds in Table 2 have some capacity for binding to proteins, although azide ions are generally known for their ability to form complexes with heavy metals. Diethyldithiocarbamate, which forms very stable metal complexes, has a similar structure to 2-ethylbutyric acid, and absorption on to the protein may be by a similar mechanism, although *NN*-dimethylglycine, with a similar type of structure to diethyldithiocarbamate, had no activity under the test conditions. The greater effectiveness of diethyldithiocarbamate, compared with 2-ethylbutyrate, may be attributed to the ability of the former to bind metal ions. Generally, non-ionic complexing agents showed no activity, but *o*-phenanthroline in combination with 0.3м-NaCl released a small quantity of DNA.

The decreased activity of *m*-hydroxybenzoate and inactivity of *p*-hydroxybenzoate compared with salicylate may also be related to the ability of the last of these compounds to form metal complexes. Evidence of the differential interaction of isomeric compounds with proteins is somewhat contradictory in that *o*-hydroxyphenylacetate had a greater affinity for albumin than the *p*-compound (Luck & Schmidt, 1948), whereas more *o*-nitrophenol than *p*-nitrophenol was absorbed by albumin (Teresi & Luck, 1948).

The inactivity of the naphthalene disulphonic acids is probably related to the highly charged hydrophilic groups at opposite ends of the molecule, which could have prevented absorption on the nucleoprotein. Orange II was the only disulphonic acid with any activity, and this compound possessed lipophilic benzeneazo and a potentially complexing *o*-hydroxyazo grouping. The dispositions of the highly charged groups could also explain the decreased activity of sulphosalicylic acid compared with salicylic acid, and the inactivity of ethylenediaminetetra-acetic acid compared with 1:2-diaminocyclohexane-*NNN'N'*-tetracarboxylate.

Direct proof of the presence of metal ions in nucleoproteins is difficult to obtain, since nucleoproteins are not easy to characterize and, being insoluble, have the capacity for ion exchange.

That transition-group metals could promote the absorption of DNA by an insoluble protein was shown by measuring the uptake of DNA by collagen which had been treated with various metals Practically no DNA was absorbed by collagen treated with Na⁺ or K⁺ ions, but approximately 30-50% was absorbed when the collagen had been treated with Cu²⁺, Co²⁺, Mn²⁺, Fe²⁺ or Zn²⁺ ions. The metal ions are almost certainly taken up by the .... of

phosphate to a carboxylate grouping through ... metal ion or to a purine or pyrimidine chelating on to

chromatography, was found to be very suitable, and a diagram of the amino acids separated by this system is shown in Fig. 1. Lysine and arginine were estimated after single-way chromatography. Estimations were made with the coloured ninhydrin-copper complexes of the amino acids (cf. Fischer & Dörfel, 1953).

The analytical figures for the amino acids present in rat-liver DNA prepared by treatment with sodium benzoate (0·3M) are shown in Table 5. The molar ratios of the amino acids present in rat-liver histone (Branish, Farley & Luck, 1951) are included for comparison. Other amino acids which were also detected were valine, methionine, alanine, serine and threonine. The glycine spot was large and tended to trail into the serine and threonine spots. The essential difference between the results and those described previously for rat-liver nucleohistone was that there was relatively less lysine and arginine compared with aspartic and glutamic acids.

Calf-thymus DNA, prepared by the sodium dodecyl sulphate method, has also been hydrolysed and the amino acids were separated and estimated. The results are shown in Table 6. Although the results are probably not of a very high degree of accuracy, the amino acids attached to the DNA bore little relation to those in calf-thumus histone
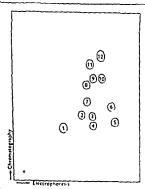
(Tristram, 1953), and the relatively high content of acidic and basic amino acids was noteworthy.

The results with herring-sperm DNA were surprising in that lysine, aspartic acid, glutamic acid and phenylalanine were present. These amino acids are absent from herring-sperm protamine. The results for herring-sperm DNA are shown in Table 7.

Rat-liver DNA prepared by treatment with 0·3M-sodium p-aminosalicylate and phenol had only traces of amino acids. No ninhydrin colours developed before about 40 hr. after spraying, and only lysine, arginine, glutamic and aspartic acids could be recognized The amounts were too small to measure accurately but indicated about a mole of amino acid in a particle weight of 1–2 × 10⁶ DNA.

Table 5. *Amino acid analyses of rat-liver deoxyribonucleic acids prepared by the use of sodium benzoate and phenol*

Amino acids are expressed as a percentage of the DNA. Molar ratios were calculated assuming that leucine = 20.

	Amount	Molar ratios	Molar ratios of rat-liver histone
Lysine	0 22	9	14
Arginine	0·14	4	16
Aspartic acid	0 20	9	9
Glutamic acid	0 20	8	12
Tyrosine	0 09	3	5
Phenylalanine	0 11	4	3
Leucine/isoleucine	0·43	20	22

Table 6. *Amino acid analyses of calf-thymus deoxyribonucleic acids*

Amino acids are expressed as a percentage of the DNA. Molar ratios were calculated assuming that leucine = 30.

	Amount	Molar ratios	Molar ratios of calf-thymus histone
Lysine	0 20	29	11
Arginine	0·10	9	15
Aspartic acid	0·23	34	7
Glutamic acid	0 14	20	5
Phenylalanine	0 01	1	4
Leucine/isoleucine	0 20	30	30

Fig. 1. Appearance of amino acids after chromatography and electrophoresis. 1, Aspartic acid; 2, glutamic acid; 3, threonine; 4, serine; 5, glycine; 6, alanine; 7, proline; 8, tyrosine; 9, methionine; 10, valine; 11, phenylalanine; 12, leucine/isoleucine.

Table 7. *Amino acid analysis of herring-sperm deoxyribonucleic acids*

Results are expressed as a percentage of the DNA

Lysine	0 09
Arginine	0 04
Aspartic acid	0 03
Glutamic acid	0 02
Phenylalanine	0 05
Leucine, isoleucine	0 20

value is an indication that the DNA is no further degraded than by other methods of isolation.

Whatever the explanation of the differential release of RNA and DNA from tissues, the fact that no DNA is released until certain salts are present before the phenol is added suggests a fundamentally different type of bonding between each nucleic acid and its respective protein. It is suggested that RNA is bound mainly by hydrogen bonds between the bases and the proteins, whereas DNA is bound by electrovalent and possibly co-ordinated metal linkages.

The actions of ribonuclease and deoxyribonuclease on RNA and DNA is an indication of a difference in the interaction of the nucleic acids with proteins. Ribonuclease acts directly upon its substrate whereas deoxyribonuclease has no activity upon DNA until activated by bivalent cations, the most active of these being Mg^{2+}, Mn^{2+}, Fe^{2+} and Co^{2+} ions (Schmidt, 1955).

The liberation of DNA by reaction with a salt (particularly sorbate and decanoate) and phenol has an interesting correlation with work on the bacteriophage. Jesaitis & Goebel (1955) have recently reported that the DNA of T_4 bacteriophage of phase II *Shigella sonnei* was liberated by a specific lipocarbohydrate present in the host. The lipocarbohydrate was fractionated into a lipid cofactor, which is probably palmitic acid, and an extracted lipocarbohydrate. Neither the lipid cofactor nor the extracted lipocarbohydrate alone released DNA from the bacteriophage but combined treatment, nor did addition of palmitic acid to the extracted lipocarbohydrate bring about release of DNA.

Linkage of DNA to a protein through metal ions (Ca^{2+} or Mg^{2+} ions) has been suggested previously by Mazia (1954) and Levine (1955) for *Drosophila melanogaster*, and by Steffenson (1955) for *Tradescantia*, mainly from genetic evidence. Hyde (1955) has commented on the action of ethylenediaminetetra-acetate in the presence of $CaCl_2$ and $MgCl_2$ on onion-root tip and *Vicia faba* chromosomes, and Eversol & Tatum (1956) have noted that ethylenediaminetetra-acetate or manganous chloride increased the frequency of crossing over of mutant strains of *Chlamydomonas reinhardi*. The motility of starfish sperm was increased by ethylenediaminetetra-acetate and also by Ni^{2+} and Co^{2+} ions (Metz & Birky, 1955), and Panzek (1956) showed that a nutritional deficiency of Zn^{2+} ion caused injury to the testis. Mann (1945) analysed ram spermatozoa and showed that iron, copper and zinc were present in greater amounts in the sperm heads than in the mid-pieces and tails. White (1955) had published the spermicidal activities of a number of chelating agents, and among the more active compounds of those tested were cupferron, sodium diethyldithiocarbamate, 1-nitroso-2-naphthol and o-phenanthroline. 1-Nitroso-2-naphthol-3,6-disulphonate ......

... . may not be directly related to the bonding of DNA to proteins, there is an interesting parallel, if not a complete correlation, in the activities of the compounds tested.

SUMMARY

1. Deoxyribonucleic acids (DNA) can be liberated from mammalian tissues by the action of certain salt solutions and phenol. Neither alone effected the release of DNA. The effective anions have strong interactions with proteins or the ability to form metal complexes.

2. DNA can be freed completely from ribonucleic acids (RNA) by treatment with ribonuclease and precipitation with 2-ethoxyethanol.

3. DNA can be freed completely from protein by the use of p-aminosalicylate and phenol and this has been developed into a preparative method yielding DNA with $\epsilon_{(p)}$ value 6340.

4. The amino acids which remain bound to rat-liver DNA prepared with sodium benzoate bore some similarities to those in rat-liver histone, but fewer basic amino acids were present.

5. Calf-thymus DNA and herring-sperm DNA prepared with the use of sodium dodecyl sulphate had considerably different ratios of amino acids from those present in their respective histone and protamine.

6. DNA was absorbed by collagen treated with Cu^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} ions but not by collagen treated with Na^+ or K^+ ions.

REFERENCES

Brunish, R., Farley, D. & Luck, J. M. (1951). *Nature, Lond.*, 168, 82.

Chargaff, E. (1955) In *The Nucleic Acids*, vol 1, p. 308. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Dische, Z. (1930). *Mikrochemie*, 8, 4.

Dunn, D. B. (1955) *Biochim biophys. Acta*, 18, 317.

Eversol, R. A. & Tatum, E. L. (1956). *Proc. nat. Acad. Sci., Wash.*, 42, 68.

a metal–carboxylate grouping as shown in Fig. 2 (cf. Wallenfels, 1955).

Histological evidence was that tissues treated with isotonic solutions of some of the salts in Table 2, or with phenol, showed no change, but that combined treatment always resulted in extraction of DNA from rat-liver nuclei. Sections of the testis showed that only the vesicular nuclei (spermatogonia, spermatocytes and spermatids) were Feulgennegative, whereas sperm heads remained Feulgenpositive. All the DNA was removed from the sperm heads when the testis was homogenized; the sperm heads were separated by centrifuging and given a longer treatment with the salt solution before the addition of the phenol. The sperm heads remained intact and cytologically recognizable after treatment with azide, cupferron or salicylate in conjunction with phenol.

Linkage of a protein to DNA by electrovalent bonds only would appear to provide little opportunity for specificity in the sequence of amino acids in the proteins. If, however, a number of electrovalent linkages were to be followed by a co-ordinate link of the type shown in Fig 2 (for example), then a much greater specificity of amino acid sequence would be required in order to complete the bonding along any given polynucleotide chain, and the positions of the aspartic and glutamic acid residues would be of considerable importance.

The activities of benzoate and sorbate were noteworthy in that these compounds have little, if any, complexing abilities. The aqueous layer from the extract of liver with benzoate and phenol was much more viscous than that from trichloroacetate and p-aminosalicylate. This greater viscosity was not due to a higher yield of DNA, but was probably related to the presence of protein in the DNA prepared with the aid of benzoate. The protein content was higher in this preparation than in those with trichloroacetate and p-aminosalicylate, and this variation in protein content with the mode of preparation could well explain the considerable

variations in the molecular weights reported for different samples of DNA.

Analyses of the protein still attached to the DNA showed many similarities to the rat-liver histone separated by Brunish et al. (1951), the main difference being a decrease in the basic amino acids. Hydrolytic experiments on calf-thymus and herring-sperm DNA, both prepared by the sodium dodecyl sulphate method, have shown protein to be present, but here there were few resemblances between these amino acids and the composition of the histone and protamine respectively. Calf-thymus DNA had relatively more basic and acidic amino acids, whereas herring-sperm DNA produced, surprisingly, amino acids which are not present in herring-sperm protamine: lysine, glutamic and aspartic acids and phenylalanine. These amino acids must be examined in further preparations but these results are of considerable significance for the bonding of proteins to nucleic acids.

Proteins of an acidic nature have been extracted from nuclei previously. Stedman & Stedman (1943, 1947) isolated a protein, chromosomin, from nuclei of various tissues and found that it was characterized by high proportions of tryptophan and glutamic acid. Wang, Mayer & Thomas (1953) extracted from rat-liver nuclei a lipoprotein which was soluble in alkali and precipitated by addition of acetic acid. They believed the material may have been a portion of the nuclear membrane. The 'residue protein fraction' of Mirsky & Ris (1951) also differed from histones, which these authors claimed could be removed from the nucleus without altering the appearance of the chromosomes. The relationships of these proteins to those found firmly bonded to DNA in the present work is not clear at the moment, but all the evidence points to considerable complexity in the components of cell nuclei.

It is clear that when tissues are treated with 0·15M-salt solution and phenol, a DNA–protein complex is liberated, and the same is probably true to some extent of the method with sodium dodecyl sulphate. The firm binding of this protein moiety, the high content of glutamic and aspartic acids and the complete removal of these by p-aminosalicylate are further indications that the protein is not bound to the DNA solely by electrovalent linkages, and that bonding of the DNA through a metal attached to a carboxylate group could provide an explanation of the experimental evidence.

The use of p-aminosalicylate and phenol has a number of advantages for the preparation of DNA from rat liver It is not necessary to isolate cell nuclei, a procedure that must involve some losses, deoxyribonucleases are probably inactivated in a manner similar to that for ribonuclease and the yield is good. Ribonucleotides and amino acids are completely absent from the product, and the [?]

Fig. 2. Possible means by which DNA could form a complex through a metal with a protein containing aspartic or glutamic acid residues

Histones from Normal and Malignant Cells

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The functions of the histones in the cell nucleus have been the subject of considerable speculation, prompted by the reports of the tissue specificity of the histones (Stedman & Stedman, 1950a). Although a difference between the basic protein in

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the sperm and somatic tissues of the salmon and fowl has been amply demonstrated (Stedman & Stedman, 1950a; Daly, Mirsky & Ris, 1950), differences between various somatic tissues have not been so well defined. Cruft, Mauritzen & Stedman (1954) reported that the histones from tumours had a lower isoelectric point than those

Fischer, F. G. & Dörfel, H. (1953) *Biochem. Z.* **324**, 544.

Flaske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Frick, G. (1954*a*). *Biochim. biophys. Acta*, **13**, 41.

Frick, G. (1954*b*). *Biochim. biophys. Acta*, **13**, 374.

Grassmann, W. & Deffner, G. (1953) *Hoppe-Seyl. Z.* **293**, 89.

Hyde, B. B. (1955). *Biol. Bull., Woods Hole*, **109**, 347.

Jesaitis, M. A. & Goebel, W. F. (1955) *J. exp. Med.* **102**, 733.

Jones, A. S. & Marsh, G. E. (1954). *Biochim. biophys. Acta*, **14**, 559.

Kirby, K. S. (1955). *Biochim. biophys. Acta*, **18**, 375.

Kirby, K. S. (1956*a*). *Biochem. J.* **62**, 31r.

Kirby, K. S. (1956*b*). *Biochem. J.* **64**, 405.

Levine, R. P. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 727.

Levy, H. B. & Snellbaker, L. R. F. (1955). *Arch. Biochem. Biophys.* **54**, 549.

Luck, J. D. & Schmidt, A. S. (1948). *Stanf. med. Bull* **6**, 133.

Mann, T. (1945). *Biochem. J.* **39**, 451.

Marko, A. M. & Butler, G. C. (1951). *J. biol Chem.* **190**, 165

Mazia, D. (1954) *Proc. nat. Acad. Sci., Wash.*, **40**, 521.

Metz, C. B. & Birky, C. W. (1955). *Biol. Bull., Woods Hole*, **109**, 365.

Mirsky, A. E. & Ris, H. (1951). *J. gen. Physiol.* **34**, 475.

Parizek, J. (1956). *Nature, Lond.*, **177**, 1036.

Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.

Scatchard, G. & Black, E. S. (1949). *J. phys. Chem.* **53**, 88..

Schmidt, G. (1955) In *The Nucleic Acids*, vol. 1, p. 581. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Stedman, E. & Stedman, E. (1943). *Nature, Lond.*, **152**, 267.

Stedman, E & Stedman, E. (1947). *Symp. quant. Biol.* **12**, 224.

Steffenson, D. (1955). *Proc nat. Acad. Sci., Wash.*, **41**, 155.

Steinhardt, J. (1941). *Ann. N.Y. Acad. Sci.* **41**, 287.

Teresi, J. D. & Luck, J. M. (1948). *J. biol. Chem.* **174**, 653.

Thomson, R. Y., Heagy, F. C., Hutchison, W. C. & Davidson, J. N. (1953). *Biochem. J.* **53**, 460.

Tristram, G. R. (1953). In *The Proteins*, vol. 1*a*, p. 211. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.

Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 715.

Wallenfels, K. (1955). *Angew. Chem.* **67**, 787.

Wang, T. Y., Mayer, D. T. & Thomas, L. E. (1953). *Exp. Cell Res.* **4**, 102

White, I. G. (1955). *Aust. J. biol. Sci.* **8**, 387.

Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

(Fig. 4). A chromatogram of a Landschütz ascites-tumour extract was very similar (Davison, 1957). No characteristic features were detected in which the tumour-protein preparations differed from those from normal tissues.

The liver and kidney preparations mentioned above showed only three peaks, and the diagrams appeared to resemble closely those from calf-thymus histones (Davison, 1957). This suggests that the calf-thymus histones and the pure mouse histones have very similar chromatographic

Fig. 3. Ascending diagram of Walker carcinoma histone of the rat, under electrophoresis at pH 8·76. (Ionic strength 0·2, 15 mA, 80v, 140 min.)

Fig. 1. Ascending (*a*) and descending (*b*) diagrams of Ehrlich ascites-histone no. 2 during electrophoresis at pH 4·60 (ionic strength 0·2, 18 mA, 105v, 2 hr.).

Fig. 2. Ascending diagrams of (*a*) calf-thymus histone and (*b*) mouse Ehrlich ascites-histone no. 3, during electrophoresis at pH 4·38. (Ionic strength 0·2, (*a*) 15 mA, 80v, 3 hr.; (*b*) 15 mA, 72v, 2 hr.)
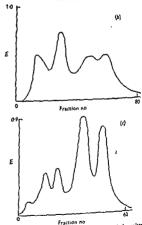
Fig. 4. Elution diagrams at pH 6·5 of (*a*) Ehrlich ascites no. 4 supernatant, (*b*) mouse-liver no. 1 supernatant, and (*c*) mouse-spleen no. 2 supernatant.

from normal tissues, as a result of which they precipitated at pH 7.

In the experiments reported below, the histones from normal and ascites-tumour cells have been compared. A preliminary report of this work was given at an informal discussion of the Faraday Society, 12 October 1956.

EXPERIMENTAL AND RESULTS

Tissues. For the isolation of cell nuclei, the livers, kidneys and spleens of 20–40 pure-line mice (C_3Hb) were used in each experiment. They were stored in solid carbon dioxide for up to 1 week before treatment. Ehrlich and Landschutz ascites cells were transplanted into the same line of mice and the cells were harvested after 5–7 days. By using ascites cells the danger of including necrotic tissue in the preparation was avoided. Although the ascitic fluid occasionally contained a considerable number of erythrocytes, the percentage of nucleated non-tumour cells was low (about 10%). A few preparations from Walker carcinoma tissue of the rat were studied.

Isolation of cell nuclei. The cell nuclei were isolated from the tissues in dilute acetic acid (Stedman & Stedman, 1950b) or 0·005M-citric acid. In both procedures the tissues were initially homogenized for 30 sec. in a Waring Blendor and the homogenate was filtered through 120-mesh metal gauze. The nuclei were separated from the cell debris by centrifuging and the course of the purification was followed by light microscopy, the orcein-fast green stain (Kurnick & Ris, 1948) being used. Samples of several preparations were fixed in buffered osmic acid, embedded in butyl methacrylate and sectioned for examination in the electron microscope (Davison & Mercer, 1956).

Some preparations of nuclei were dried by successive washes in alcohol, acetone and ether.

Extraction of the histones. To extract the histones, the nuclei were ground in a ball mill at 0° with 0·25N-HCl for 3–16 hr. The debris was centrifuged down, and the supernatant was dialysed against water or 0·5N-acetic acid, at 0°. The extracts were freeze-dried or preserved frozen at −30°.

A list of the preparations from mouse tissues is given in Table 1. The proportion of the nuclear nitrogen recovered in the histone extracts is indicated. When the acid extracts were dialysed to pH 6·5 a precipitate formed, as described

by Cruft *et al.* (1954): the fraction insoluble at pH 6·5 will be referred to as the 'neutral precipitate', and the percentage of the total acid extract which this comprised is also listed in Table 1. The proteins in the supernatant at pH 6·5, which in every case studied formed the greater proportion of the extracted protein, will be referred to as the 'neutral supernatant'. It was found that the freeze-dried proteins became less easy to dissolve on keeping, and it was usually necessary to add 0·5N-acetic acid to achieve a clear solution. Some of the preparations could not be dissolved completely, despite the use of acid, and these were centrifuged before examination. Later preparations were preserved in 0·5N-acetic acid at −30° No quantitative analyses were made on samples which could not be completely redissolved in dilute acetic acid.

Electrophoresis. The proteins were dissolved to give a 0·7–1·0% solution, and the solutions were dialysed overnight against the appropriate Miller & Golder (1950) buffers at 0°. Electrophoresis was performed in a Perkin-Elmer apparatus.

In every case studied the neutral supernatant fractions migrated to the cathode at all pH values up to 8. Most of the diagrams resembled those from calf-thymus histone, i.e. two peaks below pH 6 and two or three above (Figs. 1, 2). The Walker carcinoma histone of the rat also moved to the cathode at pH 8·76 (Fig. 3). No experiments were performed above pH 9 since the solutions became cloudy and commenced to precipitate.

[. . . .]

proteins in the preparations.

Chromatography. The histones were chromatographed on carboxymethylcellulose columns (Peterson & Sober, 1956). The adsorbent was buffered at pH 6·5 and the proteins were eluted with a NaCl concentration gradient (Davison, 1957) The distribution of the proteins in the effluent fractions was determined by the absorption of the solutions at 2780Å or by the reaction with ninhydrin (Moore & Stein, 1954).

Except for the liver no. 2 and the kidney no. 1 preparations, all the neutral supernatant solutions listed in Table·1 gave elution diagrams (measured by the ninhydrin reagent) containing four major peaks more or less clearly resolved. A small unretarded peak could also be resolved if the application of the concentration gradient was delayed. In different preparations the relative sizes of the peaks varied

Table 1. *Mouse-histone preparations studied*

Tissue		Nuclei isolation medium	Nuclear nitrogen recovered in histone extract (%)	Histone extract precipitated at pH 6·5 (%)
Ehrlich ascites tumour	1	Acetic acid	15	—
	2	Citric acid	10	27
	3	Citric acid	24	25
	4	Acetic acid	—	—
Landschutz ascites tumour	1	Acetic acid	27	23
	2	Acetic acid	33	11
Liver	1	Citric acid	21	—
	2	Acetic acid	34	8
Spleen	1	Citric acid	26	—
	2	Acetic acid	—	—
Kidney	1	Acetic acid	29	20

DISCUSSION

From these experiments it is apparent that ascites-tumour cells contain histones which are similar in electrophoretic and chromatographic behaviour to those from normal mouse tissues. The electrophoretic behaviour of the Walker carcinoma histones of the rat was also unexceptional, and resembled that of the histone from calf thymus. Thus the results obtained by this comparative study do not agree with the report of Cruft et al. (1954), who found the whole of the malignant histones which they extracted to be qualitatively distinguished from the normal by their low isoelectric points.

Apart from the first two preparations of tumour nuclei, no consistent difference was noted between the proportions of normal or tumour nuclei which could be extracted with acid. This is in agreement with the preliminary report published by Allfrey, Mirsky & Stern (1955), who found the histone content of normal and malignant leucocytes to be the same. However, Stedman & Stedman (1947) and Hamer (1951) have reported that tumour cells have a low histone content. This discrepancy may be due to the fact that these latter authors used more dilute acid for the extraction of the histones.

Although it is not claimed that the basic and acid proteins isolated from the 'neutral precipitate' are homogeneous, it is felt that the resolution into an acid and a basic fraction suggests that the precipitate may result from the aggregation of the histones in the extract with contaminating acid proteins. This would be expected to occur as the pH of the solution was taken above the isoelectric point of the acid proteins. The small proportion of the neutral precipitate which could not be re-dissolved has a composition characteristic of neither the acid nor the basic components; it may be some contaminant which has become denatured.

The contamination of the acid extracts with non-basic proteins is not surprising. Davison & Mercer (1956) have shown that nuclei isolated in aqueous media have protein (presumably cytoplasmic) adhering to the outside of the nuclear membrane. Adsorption within the nucleus is also possible since the membranes of isolated nuclei are permeable to protein (Anderson, 1953). It is possible that the malignant transformation is accompanied by the replacement of a normal by an abnormal histone, but the latter will apparently not be revealed by a study of the gross characteristics of the histones. It will require a more complete resolution of the individual components of the histone mixture than has yet been achieved.

SUMMARY

1. Nuclei have been isolated from mouse-ascites cells and normal tissues. From normal- and malignant-cell nuclei similar proportions of protein could be extracted by acid.

2. From most of these acid extracts a proportion of protein precipitated on dialysis to pH 6·5.

3. The proteins soluble at pH 6·5 were examined by chromatography and under electrophoresis. The greater proportion of them was histone.

4. The proteins insoluble at pH 6·5 were separated by chromatography into an acidic and a basic fraction.

REFERENCES

Allfrey, V. C., Mirsky, A. E. & Stern, M. (1955). *Advanc. Enzymol.* 16, 411.

Anderson, N. G. (1953) *Science*, 117, 517.

Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* 48, 126.

Cruft, H. J., Mauritzen, C. M. & Stedman, E. (1954). *Nature, Lond.*, 174, 580.

Daly, M. M., Mirsky, A. E. & Ris, H. (1950). *J. gen. Physiol.* 34, 439.

Davison, P. F. (1957). *Biochem. J.* 66, 708.

Davison, P. F. & Mercer, E. H. (1956) *Exp. Cell Res.* 11, 237.

Fraenkel-Conrat, H. & Singer, B. (1956) *Arch. Biochem. Biophys.* 60, 64.

Hamer, D. (1951). *Brit. J. Cancer*, 5, 130

Kurnick, N. B. & Ris, M. (1948). *Stain Tech.* 23, 17.

Levy, A. L. (1954) *Nature, Lond.*, 174, 126.

Miller, G. L. & Golder, R. (1950) *Arch. Biochem. Biophys.* 29, 420.

Mirsky, A. E. & Pollister, A. W. (1946) *J. gen. Physiol.* 34, 117.

Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* 211, 907.

Peterson, E. A. & Sober, H. A. (1956) *J. Amer. chem. Soc.* 78, 751.

Stedman, E. & Stedman, E. (1947) *Sym. Soc. exp. Biol.* 1, 232.

Stedman, E. & Stedman, E. (1950a). *Nature, Lond.*, 166, 780.

Stedman, E. & Stedman, E. (1950b). *Biochem. J.* 47, 508

properties, and the added complexity in most of the diagrams is due to the presence of *non-basic* proteins in the extracts. The fact that the electrophoresis studies showed the major part of all the neutral supernatants to migrate like histones is not belied by the large size of the chromatographic peaks which precede the two characteristic histone peaks emerging last (e.g. Fig. 4a). The leading components may be complexes between histones and other proteins, and in any case the ninhydrin colour yield of a protein is not proportional to the weight of the protein.

Analysis: comparison of neutral precipitate and supernatant. The Ehrlich ascites-histone no. 3 was dialysed to pH 6·5 and the precipitate and supernatant proteins were separated. They were hydrolysed in sealed tubes with 6N-HCl at 110° for 24 hr. The amino acid compositions of the hydrolysates were determined by the fluorodinitrobenzene method of Levy (1954), modified by employing the coupling conditions suggested by Fraenkel-Conrat & Singer (1956) The chromatography was also modified at the recommendation of Dr D. M. P. Phillips by replacing the Whatman no. 1 by no. 7 paper, and by using *tert.*-amyl alcohol saturated with buffer, pH 6 (Blackburn & Lowther, 1951), in place of xylene-ammonia as the first solvent. The latter was inconvenient in a laboratory where ninhydrin was being used

The amino acid compositions of the neutral precipitate and supernatant fractions are given in Table 2 Each analysis is the mean of four determinations. Arginine and histidine were not measured The proportions are not corrected for any destruction of the amino acids during hydrolysis.

The substantially higher proportion of the dicarboxylic acids in the precipitate was not unexpected, as the high lysine content suggested that some histone might be present in the precipitate as well as in the supernatant.

Neutral precipitate. The neutral precipitate from both tumour and normal tissue extracts could be redissolved almost completely in 0 5N-acetic acid. The pH of this solution could be adjusted to 4 before reprecipitation commenced. Exploratory experiments showed that this solution contained two fractions which could be separated by chromatography. The neutral precipitate from the Landschütz ascites-extract no. 2 was shaken in 0 5N-acetic acid and the fraction which remained insoluble was centrifuged off (F_1). The supernatant solution was dialysed to pH 3 and chromatographed on a carboxymethylcellulose column buffered at the same pH. The absorption of the effluent solutions at 2780Å was measured and the elution diagram was plotted (Fig. 5). The first peak was due to low-molecular-weight material. The fractions corresponding to the two major peaks (respectively F_2 and F_3) were grouped, the pH was adjusted to 10 with NaOH, and 3 vol. of acetone were added After standing overnight at 0° the precipitates which had formed *were centrifuged* down and hydrolysed The amino acid composition of each fraction was determined by the fluorodinitrobenzene method The results are shown in Table 3. F_1, F_2 and F_3 were present in the approximate ratios of 2.1 6, by wt It is doubtful, however, if the whole of F_2 was precipitated in the alkaline conditions employed

An attempt was made to precipitate the non-histone component of the neutral precipitate by using 0 34N-$HgSO_4$ in 88N-H_2SO_4 (Mirsky & Pollister, 1946) Although a precipitate containing more dicarboxylic residue than

basic residues was obtained, it represented only a very small fraction of the total protein. This would suggest that some of the acid material is of relatively low molecular weight.

Table 2. *Amino acid composition of neutral precipitate and supernatant from acid extract of ascites-tumour cells*

Composition is expressed in molar ratios (no. of residues/ 100 residues). Arginine and histidine were not determined.

	Supernatant	Precipitate
Alanine	12·5	10 3
Aspartic acid } Glutamic acid }	17·9	25 6
Cystine	0·2	0 3
Glycine	9 6	8·4
Leucine } Isoleucine }	15 0	14·5
Lysine	14 4	10 7
Phenylalanine	2 8	3 0
Proline	4 7	3·9
Serine	6 8	8·1
Threonine	6 8	6·4
Tyrosine	2·4	2·6
Valine	7 0	7·1
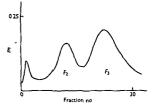
Fig. 5. Elution diagram of Landschutz ascites 'neutral precipitate' chromatographed at pH 3 1.

Table 3. *Amino acid composition of fractions from 'neutral precipitate'*

Fractions were obtained from Landschutz ascites-tumour extract. Composition is expressed in molar ratios (no. of residues/100 residues)

	F_1	F_2	F_3
Alanine	13 3	9 75	12·4
Arginine	Low	0 35	9·4
Aspartic acid	8 1	12 6	5·8
Glutamic acid	11 7	11 6	9 8
Glycine	11·2	11·1	9 5
Histidine	1 6	2 4	1·5
Leucine } Isoleucine }	15 6	15 5	13 8
Lysine	11 0	9·3	10 5
Phenylalanine	2 5	3 3	2 3
Proline	3 0	2 4	3 9
Serine	6 6	9 0	7 4
Threonine	7·8	5 9	6 9
Tyrosine	1·5	2 0	1·3
Valine	6 1	5 6	5·7

0·1 or 0·05 M-buffers; when the solution had passed into the column more of this buffer was added to wash the protein into the adsorbent. The column reservoir was filled with buffer and a concentration gradient in the eluting solutions was then produced by adding at a controlled rate a solution of the same buffer containing 0·9 M-NaCl. Mixing of the solutions in the reservoir was effected by a magnetic stirrer.

An alternative device used on the small columns consisted of an open-topped Perspex box with a diagonal diaphragm of stainless steel sealed in with wax (Fig. 1). For low rates of flow the two solutions merging in the three-way tap mixed satisfactorily by diffusion alone. The shape of the concentration gradient could be modified by bending the diaphragm.

Effluent analysis. The concentration of protein in the column effluents was measured by the absorption of the solutions at 2780 Å, or by the ninhydrin reagent (Moore & Stein, 1954).

Effect of pH. The chromatographic behaviour of the histones was studied over a pH range 3·0–8·1. Sodium phosphate, citrate and acetate buffers (0·05 or 0·1 M) were used.

At pH 3–4·5 two main components could be distinguished in the effluent diagrams. These were preceded by a smaller factor .... (T... C... li... hh...)..... Hi.....

complexes, as well as homogeneous proteins, may be chromatographed as distinct peaks, and since it is known that the histones readily aggregate above pH 7, the delineation of five peaks on the diagram does not necessarily imply the presence of five distinguishable proteins.

Recovery of the proteins from the adsorbent. In preliminary experiments (Davison & Shooter, 1956) the proportion of the protein recovered from the columns was determined by the colour given with ninhydrin, by the protein applied to the column and that in the total column effluent. Recoveries calculated in this way for duplicate chromatograms were frequently found to differ widely. The cause of this behaviour was not determined, but from subsequent observations it is possible that varying amounts of degradation occurred in the solutions as they stood on the fraction collector. An increase of amino end-groups would increase the ninhydrin colour.

In subsequent experiments the protein recovery was calculated from the nitrogen content of the effluents. It was found that, contrary to our earlier report, the proportion of the protein eluted from the columns decreased as the pH was raised. It was also found that the recovery from the columns decreased as the temperature was raised, although the elution diagrams were not qualitatively changed between 0° and 50°. The effect of temperature and pH on nitrogen recovery is shown in Table 1.

Isolation of proteins from column effluents. The concentration of the proteins in the column effluents was often very low. Dialysis of the pooled fractions, followed by freeze-drying, was inefficient, and not more than 70% of the applied protein was ever recovered in this way. Precipitation of the proteins by phosphomolybdic acid or perfluoro-octanoic acid was effective, but there remained the difficulty of removing the precipitants. The most efficient method of recovery was to adjust the solutions to pH 10 and to add 3 vol. of acetone. After standing for 24 hr. at 0° approx. 80% of the histones could be precipitated from solutions

as dilute as 0·002%. The precipitates were washed in 95% acetone to free them of salt. This procedure precluded the use of sodium citrate and phosphate buffers since these are insoluble in 75% acetone. Up to 95% recoveries of the chromatographed histones were obtained in this way.

Analysis. Nitrogen determinations were made by the micro-Kjeldahl method. Amino acid analyses were made by the fluorodinitrobenzene method of Levy (1954). The procedure was slightly modified in detail (Davison, 1957).

The method of Moore & Stein (private communication) was used to determine accurately the ratio of the basic
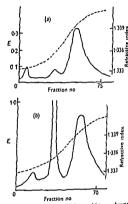
Fig. 2. Elution diagrams of calf-thymus histones chromatographed at pH 4·0. Protein concentration was measured .... fractive index.

| | At pH 8·1 | | At 4° |
Temp	Recovery (%)	pH	Recovery (%)
4°	(i) 84	4·1	(i) 94
	(ii) 87	5·0	(i) 90
20	(i) 75	6·6	(i) 97
	(ii) 73		(ii) 92
50	(i) 54	8·1	(i) 84
			(ii) 87

Chromatography of Histones

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Crampton, Moore & Stein (1955) made a partial separation of the histones of calf thymus and other tissues by chromatography on IRC-50 resin. With a progressively increasing concentration of Ba^{2+} or Ca^{2+} ions they were able to elute three proteins, richer in lysine than in arginine, which together comprised about 40 % of the histones applied to the columns. They found that the corresponding proteins from different calf tissues had very similar amino acid compositions. Cruft, Mauritzen & Stedman (1954) have identified three electrophoretic components in the histones of ox thymus and liver, and they found that the major or β-histones from these two tissues differ in electrophoretic mobility. This they regard as an indication of the tissue specificity of the histones. These β-histones readily aggregate, and Davison & Shooter (1956) have shown that the aggregating histones are those rich in arginine. Thus the evidence from electrophoresis does not conflict with the results of Crampton et al., which seemed to demonstrate no cell specificity in the lysine-rich histones. However, the electrophoretic evidence cannot be accepted as conclusive since any small amount of non-histone contaminants loosely combined with these basic proteins might change the electrophoretic mobility. To test this question of tissue specificity by the analysis of different fractions it is necessary to find a chromatographic system capable of separating the arginine-rich as well as the lysine-rich histones. The experiments to be reported here have shown that the histones can be completely eluted from carboxymethylcellulose, although they are not well separated. A preliminary report on this chromatographic system has been published (Davison & Shooter, 1956).

EXPERIMENTAL

Adsorbent. Carboxymethylcellulose was prepared in the manner described by Peterson & Sober (1956) The dried white powder was dispersed in the appropriate buffer solutions with rapid stirring and the slurry was then poured into chromatographic columns A thin layer of acid-washed Celite 545 (Johns-Manville Co. Ltd.) helped to prevent the adsorbent clogging the sintered glass plate at the bottom of

the columns. The carboxymethylcellulose was allowed to settle under gravity to give a column 10–15 cm high Tubes with an internal diam. of 1 cm. were used for qualitative studies, with 5–10 mg. of protein. Fifty to 100 2 ml. fractions were sufficient to delineate the effluent diagrams. For preparative studies a 1·4 cm. diam. column was used, with a loading of up to 120 mg. of protein. Larger columns were used for some experiments but, unless the adsorbent was initially suspended in butanol, even packing was difficult to achieve. The linearity of the front was checked by passing a small volume of potassium chromate through the column

The flow rates were reduced to 4–8 ml /cm³ cross-section, by fixing drawn-out capillary tips to the columns.

Proteins. The histones used in the exploratory studies were those from calf thymus, prepared as previously described (Davison, James, Shooter & Butler, 1954). In later experiments some histones extracted from mouse-cell nuclei by acid were chromatographed. The cell nuclei were isolated from various mouse tissues in dilute acid (Davison, 1957).

Gradient devices. The proteins were eluted from the adsorbent by buffered solutions of progressively increasing ionic strength. The proteins were applied to the columns in
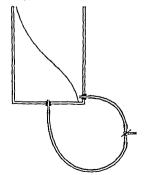
Fig 1. Gradient device to provide continuously changing eluting solution. The more concentrated, denser solution is placed in the left-hand reservoir.

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suggested this protein was rich in dicarboxylic acids, and this was confirmed by an analysis of one sample by the Levy (1954) method (see Table 2). This acid protein comprised less than 3% of the total histone.

It was noticed some years ago in these laboratories that a few thymus-histone preparations, on electrophoresis at pH 4·5, showed a very small component migrating behind the histone boundaries. It is possible that this was the acid protein.

The second fraction separated at pH 4·2 gave a high colour yield with ninhydrin, but had a low absorption at 2780λ: it consisted solely of the lysine-rich histone (Davison & Butler, 1954; Crampton et al. 1955). It comprised 11–13% of the various histone preparations analysed.

The third and major peak comprised 80% of the total histone and contained more than one species of protein. Samples were recovered from different portions of the third peak and analysed for basic amino acids by the method of Moore & Stein (1956, private communication). The molar ratio of lysine:arginine diminished from approx. 1·5 to less than 1·0, from the leading edge to the trailing edge of the peak. Some results of analyses are summarized in Table 3.

From columns buffered between pH 5 and 6 5 a further component could be isolated. This emerged with the first application of the gradient, ahead of

Table 3. *Composition of fractions from the major peak from calf-thymus histone*

The analysis is expressed as molar ratios relative to lysine.

	Original histone	Leading half	Final third	Final fifth
Lysine	1·0	1 0	1·0	1 0
Arginine	0 54	0 66	0 83	0 94
Histidine	0 20	0 18	0 21	0 25
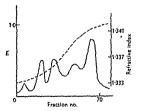
Fig. 5. Elution diagram of histone extract from mouse Landschütz ascites-tumour cells chromatographed at pH 6 5. The broken line indicates the refractive index of the eluting solution.

the lysine-rich component. If the eluting gradient were applied to the column immediately after the protein, this intermediate peak could not be distinguished from the small . . . .

lower pH in the two main peaks.

Above pH 6·5 more intermediate peaks appeared and the two main peaks decreased in size: this behaviour probably reflected further aggregate formation.

Histones extracted from a variety of calf and mouse tissues were examined qualitatively on the carboxymethylcellulose columns at pH 6·5 (see also Davison, 1957). One preparation from mouse kidneys and one from mouse livers gave elution diagrams essentially similar to those from calf-thymus histones (Fig. 4). In most diagrams, however, five peaks could be detected in proportions which varied from one preparation to another (Fig. 5). It is probable that this added complexity arose from non-histone proteins in the extracts. The presence of such contaminants in some preparations was confirmed by electrophoresis at pH 2·2: at this pH the different proteins do not appear to aggregate, and a small peak could be detected trailing the sharp histone boundary.

The two peaks which were eluted last from these pH 6 5 columns appeared in every diagram at the same position and were presumed to correspond to the two major components resolved at pH 4·2. The presence of two or more peaks preceding these was taken to indicate contaminants. Thus the columns at pH 6 5 were mainly of value in assessing the purity of the histone preparations. For an efficient fractionation the adsorbent was buffered at a lower pH, where the protein interactions were minimized. It was found possible to operate these columns at pH 3·1, but the proteins were then eluted at lower salt concentrations.

Chromatography on IRC-50. The histone-elution diagrams at pH 4·2 were very similar to those obtained from IRC-50 (Crampton et al. 1955). When each of the main components from the carboxymethylcellulose column was isolated and rechromatographed on IRC-50 with barium acetate the similarity was confirmed; the first, lysine-rich, histone emerging in the position of Crampton's histone A, and the second component in the position of histone B. The proportion of the latter fraction which was eluted from IRC-50 was not determined.

DISCUSSION

The chromatographic system described here has several advantages over that devised by Crampton et al. (1955), notably in the higher recoveries from

amino acids in the histone fractions separated by chromato-graphy. Lysine, histidine, ammonia and arginine were ~~~ ~~~~ ~~~~~

concentration was determined by the modified ninhydrin reagent of Moore & Stein (1954). The 2-methoxyethanol (methyl Cellosolve) in this reagent was purified before use by passing it through a layer of Zeo-Karb 225 and a column of activated alumina.

Protein hydrolysis. For the amino acid analysis the proteins were hydrolysed in sealed tubes with 6 N-HCl at 110° for 24 hr.

RESULTS

Qualitative examination of histones. For quali-tative examination of the histone preparations the carboxymethylcellulose columns were buffered at pH 6·5. Typical elution diagrams are shown in Figs. 3 and 4. These diagrams show more distin-guishing features than those at lower pH.

For preparative chromatography the columns were buffered at pH 4·2 with sodium acetate and acetic acid. At this pH the large-molecular-weight histone aggregates begin to dissociate (Davison *et al.* 1954) and this is obviously a prerequisite if the component proteins are to be efficiently separated.

Degradation in the histone fractions. Crampton (1956) reported that occasionally, when a histone fraction separated by chromatography was re-chromatographed, it gave rise to a fast-running component in addition to a peak moving in the original position. Similar behaviour was observed with fractions from carboxymethylcellulose. The fast-running fraction gave a much higher ninhydrin colour/mg. of nitrogen than the parent material. It was also found that the fast material could be eluted from IRC-50 resin columns with Na⁺ ions, whereas the histones can only be eluted with bivalent ions. These observations suggested that the histones were degraded either on the column or during their subsequent handling. Butler, Davison, James & Shooter (1954) have shown that histones are rather easily degraded at neutral pH. Whether the degradation is the result of proteolysis or the dissociation of complex proteins is not known. As a precaution against proteolysis the buffer solu-tions were saturated with *p*-chloromercuribenzoic acid and the chromatograms were run at 4°.

Carboxymethylcellulose buffered at pH 4·2 separ-ated calf-thymus histones into three components (Fig. 2*a, b*). The first peak passed directly through the column without the application of the gradient; since in many cases nothing could be precipitated from this effluent fraction it is probable that much of it is comprised of degraded protein. In two pre-parations, however, a precipitate was obtained. Qualitative amino acid paper chromatography
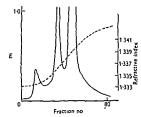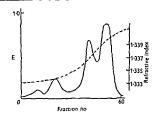
Fig. 3 Elution diagram of calf-thymus histones chromato-~~~ ~~ ~~~~ ~~~~~~ ~~~ ~~~ ~~~~~~~~~~~~~~. broken line indicates the refractive index of the eluting solution.

Fig. ~ ~~~~ ~~ ~~~~~~~~ ~~ ~~~~~~ ~~~~~~ ~~~~~~~ ~~~~~~~~. of the eluting solution.

Table 2. *Composition of leading fraction from calf-thymus histone chromatographed at pH 4·2 on carboxymethylcellulose*

The analysis is expressed in molar ratios (no. of amino acid residues/100 residues)

Arginine	2·7
Alanine	8·8
Aspartic acid	19·7
Glutamic acid	32·7
Glycine	8·4
Histidine	1·4
Leucine	5·0
Isoleucine	
Lysine	8·5
Proline	0·0
Serine	3·4
Threonine	3·5
Valine	5·6

The Effect of Nitrogen Mustards on the Incorporation of Amino Acids into Protein by *Staphylococcus aureus*

By A. R. CRATHORN AND G. D. HUNTER

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Nitrogen mustards have been used clinically with some success in the treatment of malignant lymphadenopathies and the chronic leukaemias (see, for instance, Galton, Israels, Nabarro & Till, 1955), and they are also effective in inhibiting the growth of some solid tumours in experimental animals. Their biochemical mode of action remains obscure, although many studies *in vitro* have shown that they readily enter into chemical combination with nucleic acids and proteins under very mild conditions (reviews by Alexander, 1954 and Ross, 1952; also Cohn & Crathorn, unpublished work). More recently, Cohn (unpublished work) has found that p-di-(2-chloroethyl)amino-DL-phenylalanine becomes firmly bound to the proteins of liver and other tissues when the compound is injected interperitoneally into rats.

The present investigation represents an attempt to determine the extent to which the nitrogen mustards exert their effects on the direct incorporation of amino acids into proteins. A bacterial system was selected because of its simplicity. The use of *Staphylococcus aureus* offered a further advantage: only in this organism have two modes of amino acid assimilation been demonstrated, one associated with net protein synthesis and the other with an 'exchange' reaction between free amino acids and the amino acid residues within the preformed protein molecules (Gale & Folkes, 1953a).

METHODS

Organism. The organism used throughout this work was *Staphylococcus aureus* strain Duncan (Gale & Folkes, 1953b), which was kindly supplied by Dr K. McQuillen. It was maintained by frequent subculture on solid peptone-agar slopes. Larger batches for experimental purposes were

Preparation of suspensions and conditions for study of uptake of ^{14}C *labelled amino acids* The bacteria were harvested after overnight growth and washed with water. Suspension densities were determined from the absorption at 420 mμ on a Unicam spectrophotometer (Model SP. 600)

previously calibrated against the organism used Thick suspensions of the organism (50 mg. dry wt /ml) were then prepared in 0 05 M-phosphate buffer (prepared from KH_2PO_4, brought to pH 7 with NaOH) containing 1% (w/v) glucose, and 5 ml. samples were pipetted into each flask; the nitrogen mustards to be studied were added to the individual flasks at this stage. After aerobic incubation, while shaking at 37° for 0 5 hr, the ^{14}C-labelled amino acid was added, and the incubation continued for a further period of 90 min. In this way the labelled amino acid was taken up under exchange conditions In experiments where an investigation was made of the effects of nitrogen mustards on uptake under conditions of net protein synthesis the bacteria were collected and transferred to an inorganic salts-glucose medium containing a complete mixture of amino acids The labelled amino acid was then added and carried out

37° for 1 hr. The nucleic acids were then removed by treatments at 95° with 5% (w/v) trichloroacetic acid. The proteins were finally washed twice with acetone and dried at 70°.

Toxicity tests. The incubations of buffered bacterial suspensions with nitrogen mustards were carried out at 37° for periods similar to those used in the examination of the uptake of amino acids by the cells. Viable cell counts were then made on peptone-agar plates after serial dilutions of the bacterial suspensions with sterile water. These dilutions had reduced the viable counts to the order of a few hundred/plate in control suspensions untreated with nitrogen mustard.

Assays of radioactivity. These were carried out on 'infinitely thick' samples of protein plated on 1 cm.[2] an EHM 2S end-window 1950 · G.E.C, London,)0C counting unit manufactured by Panax Equipment, Ltd , Mitcham, Surrey. The counter was housed in a lead castle manufactured by E R D. Ltd., Slough, Bucks Under these conditions a ^{14}C-labelled protein sample of specific activity an observed counting rate of about

P. F. DAVISON

the columns and the possibility of precipitating the histones from the effluent fractions. It does not, however, satisfactorily resolve the arginine-rich histones. It is not clear if the continuous change observed in the lysine:arginine ratio of the proteins in successive portions of the major fraction can be attributed to relatively few components emerging in overlapping bands, or to a very large number of proteins. If the histones function as gene inhibitors, as Stedman & Stedman (1950) have suggested, the latter possibility would appear more likely. However, the little evidence available suggests that at least 20% of the major histone fraction is homogeneous, i.e. the histone B of Crampton. The problem of the complexity of this major fraction is at present being pursued by a study of the distribution of the N-terminal amino acids through the chromatographic peak.

The detection of the acid protein in the calf-thymus histones exemplifies one of the fundamental difficulties: that of purifying the basic proteins. Since this contaminant was found in a preparation from such a convenient tissue as thymus, it is not surprising that higher proportions of contaminants should be found in extracts from other tissues. Many purification procedures used in the past have resulted in unintentional fractionation of the histones (Davison & Butler, 1956). The carboxymethylcellulose columns, operated at pH 4 or lower, could probably provide a simple and effective means of freeing the histones of non-basic contaminants.

Although 90–95% of the protein nitrogen was recovered from these chromatograms, it is possible that small amounts of histone were lost. These would presumably be the histones richest in arginine, since they require the higher concentrations of salt to be eluted. Such a loss could not be demonstrated by analysis.

None of the fractions listed in Table 3 has a lysine:arginine ratio less than 1·0, and of several other fractions separated by chromatography only one, from the tail of the major peak, had this ratio slightly lower (0·95). The much lower value found for the histone fraction precipitated by ammonia (Davison & Shooter, 1956) has not been found in the fractions separated by chromatography. Whether the earlier analyses were in error, or whether the two fractionation methods are resolving different species of proteins, has yet to be determined.

SUMMARY

1. A description is given of a chromatographic system capable of separating the histones of calf thymus and other tissues into the lysine-rich histone and the remainder, with a recovery higher than 90%.

2. Analysis has shown that the lysine:arginine ratio decreases in the histone eluted with higher concentrations of salt, but it could not be decided whether the histones consisted of a few limited species or were more complex.

3. Examination of the protein in the acid extracts of nuclear materials from calf thymus and other tissues has demonstrated the presence of non-basic protein contaminants.

REFERENCES

Butler, J. A. V., Davison, P. F., James, D. W. F. & Shooter, K. V. (1954). Biochim. biophys. Acta, 13, 224.

Crampton, C. F. (1956). Fed. Proc. 15, 237.

Crampton, C. F., Moore, S. & Stein, W. H. (1955). J. biol. Chem. 215, 787.

Cruft, H. J., Mauritzen, C. M. & Stedman, E. (1954). Nature, Lond. 174, 580.

Davison, P. F. (1957). Biochem J. 66, 703.

Davison, P. F. & Butler, J. A. V. (1954). Biochim. biophys. Acta, 15, 439.

Davison, P. F. & Butler, J. A. V. (1956). Biochim. biophys. Acta, 21, 568.

Davison, P. F., James, D. W. F., Shooter, K. V. & Butler, J. A. V. (1954). Biochim. biophys Acta, 15, 415.

Davison, P. F. & Shooter, K. V. (1956). Bull. Soc. chim. Belg. 65, 85.

Levy, A. L. (1954). Nature, Lond., 174, 126.

Moore, S. & Stein, W. H. (1954). J. biol Chem. 211, 907.

Peterson, E. A. & Sober, H. A. (1956). J. Amer. chem. Soc. 78, 751.

Stedman, E. & Stedman, E. (1950). Nature, Lond., 166, 780.

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The present investigation represents an attempt to determine the extent to which the nitrogen mustards exert their effects on the direct incorporation of amino acids into proteins. A bacterial system was selected because of its simplicity. The use of *Staphylococcus aureus* offered a further advantage. only in this organism have two modes of amino acid assimilation been demonstrated, one associated with net protein synthesis and the other with an 'exchange' reaction between free amino acids and the amino acid residues within the preformed protein molecules (Gale & Folkes, 1953a).

METHODS

Organism. The organism used throughout this work was *Staphylococcus aureus* strain Duncan (Gale & Folkes, 1953b), which was kindly supplied by Dr K. McQuillen. It was maintained by frequent subculture on solid peptone-agar slopes Larger batches for experimental purposes were obtained by growing the organism on a liquid medium of the following composition (%): glucose, 1; Marmite, 0 5; peptone, 1; NH₄Cl, 0 2; Na₂HPO₄, 0 6; KH₂PO₄, 0 3; NaCl, 0 3, Na₂SO₄,10H₂O, 0 025, MgCl₂,6H₂O, 0 0085 (all w/v) At 37° on this medium the yield of cells was approx 1 mg. dry wt /ml when grown for 16 hr in Roux bottles

Preparation of suspensions and conditions for study of uptake of ¹⁴C-*labelled amino acids.* The bacteria were harvested after overnight growth and washed with water Suspension densities were determined from the absorption at 420 mμ on a Unicam spectrophotometer (Model SP. 600)

previously calibrated against the organism used Thick suspensions of the organism (50 mg dry wt/ml) were then prepared in 0 05M-phosphate buffer (prepared from KH₂PO₄, brought to pH 7 with NaOH) containing 1% (w/v) glucose, and 5 ml. samples were pipetted into each flask; the nitrogen mustards to be studied were added to the individual flasks and, while shaking at 37° for 0-5 hr., the ¹⁴C-labelled amino acid was added, and the incubation continued for a further period of 90 min. In this way the labelled amino acid was taken up under exchange conditions. In experiments where an investigation was made of the effects of nitrogen mustards on uptake under conditions of net protein synthesis the bacteria were collected and transferred to an inorganic salts-glucose medium containing a complete mixture of amino acids. The labelled amino acid was then added and the incubation continued as in the experiments carried out [...]

[...] 37° for 1 hr. The nucleic acids were then removed by [...] treatments at 95° with 5% (w/v) trichloroacetic acid. The proteins were finally washed twice with acetone and dried at 70°.

Toxicity tests. The incubations of buffered bacterial suspensions with nitrogen mustards were carried out at 37° for periods similar to those used in the examination of the uptake of amino acids by the cells. Viable cell counts were [...] made on peptone-agar plates after serial dilutions of [...] plate in control suspension [...] mustard.

Assays of radioactivity. These were carried out on 'infinitely thick' samples of protein plated on 1 cm² polythene disks (Popják, 1950), an EHM 2S end-window Geiger-Müller counting tube supplied by G.E.C, London, being used in conjunction with a type 100C counting unit manufactured by Panax Equipment Ltd., Mitcham, Surrey. The counter was housed in a lead castle manufactured by E.R.D Ltd, Slough, Bucks. Under these conditions a ¹⁴C-labelled protein sample of specific activity 1 μc/g would give an observed counting rate of about 600 counts/min. when plated at 30 mg./cm² (i.e. a counting efficiency of 1·2%).

Materials. [1-¹⁴C]Glycine was supplied by the Radiochemical Centre, Amersham, Bucks. DL-[3-¹⁴C]Phenylalanine was synthesized in these laboratories by Dr V. C. E. Burnop.

Details of the syntheses of the nitrogen mustards used are to be found in the recent literature (Everett, Roberts & Ross, 1953; Bergel & Stock, 1954; Davis, Roberts & Ross, 1955; Bergel, Everett, Roberts & Ross, 1955b).

Tumour-inhibition tests. (Personal communication from Professor A. Haddow.) Tumour-growth-inhibition tests by nitrogen mustards were carried out on the transplanted Walker rat carcinoma (Badger, Elson, Haddow, Hewett & Robinson, 1942; Haddow, Harris, Kon & Roe, 1948) Most of the results quoted here have been briefly reported elsewhere (Everett *et al.* 1953; Davis *et al.* 1955; Bergel *et al* 1955b).

RESULTS

The initial experiments were carried out with three aliphatic nitrogen mustards: dimethyl-(2-chloroethyl)amine (M1), methyldi-(2-chloroethyl)amine (M2) and tri-(2-chloroethyl)amine (M3), (see Table 1). M2 is widely used in the treatment of leukaemia and allied diseases, it being often preferred to M3, which is rather more active but also more toxic than M2 (Galton, private communication) It was found (Table 1) that the inhibition of the amino acid-exchange reaction in our system paralleled the therapeutic efficiency of the compounds, the inhibition of incorporation being greater than would be explained by the reduction in viability of the organism. In a further experiment,

the uptake of amino acids by the M3-poisoned system was studied under conditions where amino acid exchange could be compared with the uptake of amino acids under conditions otherwise favourable for net protein synthesis. The inhibitions of amino acid uptake were the same in the two cases, contrary to a statement made in an initial report of this work (Crathorn & Hunter, 1956). It therefore appears that with these substances the inhibition of the exchange reaction runs parallel with the inhibition of overall protein synthesis.

In experiments with other nitrogen mustards, 'exchange' conditions were normally used, as it was possible to simplify the procedures by using thick suspensions of bacteria in relatively small volumes of buffered solutions.

The series of aromatic nitrogen mustards bearing aliphatic acid side chains was next studied, as in this series there is an interesting variation in the activities of different compounds on the Walker rat carcinoma; this series also contains one of the compounds (M5) that has been used therapeutically. Here a very close correspondence between the activities on the Walker tumour and the amino acid-exchange system was obtained (Table 2). Unfortunately, a supply of the other inactive member with normal alipatic side chains, namely *p*-di-(2-

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Table 1. *Comparison of the effectiveness of aliphatic nitrogen mustards in cancer chemotherapy with their ability to reduce the extent of the amino acid uptake in Staphylococcus aureus and their toxicity towards this organism*

Nitrogen mustards were used in final concentrations of 6·4 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was added to give a final concentration of 0 32μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g of protein.

Reference used in text.	Chemical formula of the nitrogen mustards	Effectiveness in cancer chemotherapy	Inhibition of the exchange reaction with phenylalanine (%)	Reduction in viability of the organism under similar conditions (%)
M1	$(CH_3)_2N \cdot CH_2 \cdot CH_2Cl$	—	0	0
M2	$H_3C \cdot N(CH_2 \cdot CH_2Cl)_2$	+ +	75	50
M3	$N(CH_2 \cdot CH_2Cl)_3$	+ + +	98	50

Table 2. *Comparison of the inhibitory effect of acidic aromatic nitrogen mustards on the Walker rat carcinoma with their ability to reduce the extent of amino acid uptake in Staphylococcus aureus*

Nitrogen mustards were added to give a final concentration of 3 3 mM DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/ mg) was used in a final concentration of 0 32μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx 1 μc/g. of protein.

Reference used in text	R, in chemical formula of the nitrogen mustard $(Cl \cdot CH_2 \cdot CH_2)_2N \cdot p \, C_6H_4 \cdot R$	Assessment of inhibition of Walker carcinoma	Inhibition of the exchange reaction with phenylalanine (%)
M4	$\cdot CH_2 \cdot CO_2H$	+ +	95
M5	$\cdot [CH_2]_2 \cdot CO_2H$	+ + +	97
M6	$\cdot [CH_2]_3 \cdot CO_2H$	− *	5
M7	$\cdot O \cdot [CH_2]_2 \cdot CO_2H$	+	44
M8	$\cdot O \cdot [CH_2]_3 \cdot CO_2H$	−	49

* Results of tests were variable. (Occasionally found: +.

mustards with free amino acid in the medium. Calculations based on the results of chemical experiments (Fruton, Stein & Bergmann, 1946) on the reaction of M2 with phenylalanine showed that under the experimental conditions described above not more than 0·2% of the amino acid could have reacted chemically with the nitrogen mustard, even if direct contact between the two substances had been maintained throughout the period of incubation. Further evidence supporting the view that this effect is negligible is inherent in the results (Table 2) obtained with homologues in the series of aromatic nitrogen mustards having aliphatic acid side chains. Their chemical re-activities, measured in terms of carbonium ion formation, are very similar, but their biological effects measured in the staphylococcal system show great variation.

The interpretation of these results on a chemical basis must therefore take into account the inter-action of the nitrogen mustards with the various cellular constituents. There is from these experi-ments no evidence that the nitrogen mustards actually penetrate the boundaries of the cell. It is clear that such a penetration would be followed by some reaction between the inhibitor and the cellular protein and nucleic acid, but the fact that the toxic effects of the nitrogen mustards are not marked within the short experimental period shows that the extent of the reaction cannot be sufficient to effect any important changes in the overall metabolism of the cells. However, the possibility of a specific reaction of the nitrogen mustards with a site of special importance in protein synthesis cannot be ruled out. If such a site were situated in the cell wall or cytoplasmic membrane, then the transport of amino acids into the cell could clearly be hindered; and the active transport of amino acids across cell walls and membranes must surely be envisaged as a part of the chain of events leading from amino acids to protein. To support this view, the recent work of Cowie & Walton (1956) on amino acid assimilation by *Torulopsis utilis* has shown that, in this micro-organism at least, absorbed amino acids do not exist in the free state at all but enter at once into a metabolic pool where they are associated with sites intimately concerned with protein synthesis. Certainly our results would not preclude an action of the nitrogen mustards on this first stage alone. There is a further possible explanation of our observa-tions: the amino acids in the metabolic pool may exist in local concentrations that are sufficiently high for chemical reaction with the inhibitor molecules to proceed at rates much greater than those pertaining to the extracellular concentra-tions. If these rates were high enough to prevent the incorporation of a considerable proportion of the amino acid into protein, the differential

activities of nitrogen mustards containing chloro-groups of similar chemical reactivities could be ascribed to different extents of penetration into the cell.

It is impossible to say whether the results ob-tained in this bacterial system can be correlated with results of metabolic studies on higher organisms. If the processes of protein synthesis follow similar pathways in all living cells then our results suggest that, in general, the primary inhibitory action of nitrogen mustards is to produce a block at some stage in the process of protein synthesis. On the assumption that there are certain similarities in the processes of protein synthesis in all organisms, the correlation between the results of tests on the bacterial system and of the same compounds on the Walker rat carcinoma are presented. An additional negative correlation might be mentioned at this point. The compound 1:4-dimesyloxybutane (Myl-eran) active against chronic myelogenous leukaemia is completely inactive in the bacterial system; nor does it react with amino acids or nucleic acids. Clinical observations, however, suggest that the mode of action is different from that of the nitrogen mustards.

It will be noticed (Table 4) that there are some apparent exceptions to the correlation. In particular it may be significant that the two quaternary com-pounds (M18 and M19) which showed relatively strong activity in the bacterial system are both very water-soluble. In one case at least the inactivity against the Walker carcinoma was surprising and could probably be explained by rapid excretion of the compound Very little inhibition of amino acid uptake was found when M13 was examined (Table 4), but it has not been tested against the Walker rat carcinoma except under conditions where other toxic side effects are manifest. Thus the positive reaction that we assign to it must remain in some doubt.

Finally it should be mentioned that alterations appearing in the blood picture in the course of treatment of animals with the aromatic nitrogen mustards with aliphatic acid side chains have recently been described (Elson, 1955). The correla-tions with our results and with the inhibitions of the Walker rat carcinoma are again close.

SUMMARY

1. An investigation has been made of the effects produced by several aliphatic and aromatic nitrogen mustards on the incorporation of ^{14}C-labelled amino acids into protein by *Staphylococcus aureus*

2 Different nitrogen mustards inhibited this amino acid incorporation to different extents. Where there was a pronounced inhibition, it could

published as part of a wider study of histones (Luck *et al.* 1956). However, the results obtained here are not in good agreement with those reported.

MATERIALS AND METHODS

Preparation of histones. The mixed histones were prepared from previously isolated nucleoprotein from fresh frozen calf-thymus glands by the method of Davison, James, Shooter & Butler (1954). The series of preparations used were labelled H1, H16, H17 and H18. Some fractions of the histones were also examined. Thus the sodium chloride-ammonia treatment of the histones H18 by the method of Davison & Shooter (1956) gave a histone precipitate (H18A) and soluble histones in the supernatant (H18B). The citric acid extraction of whole-thymus nucleoprotein (Davison & Butler, 1954) yielded a small amount of histone (labelled H7), which was then separated into two fractions, H7/1 and H7/2, by column chromatography on carboxymethylcellulose by the method of Davison & Shooter (1956) In addition the whole of the proteins extracted by acid from thymus glands have been examined. For this purpose, thymus gland (50 g.) was blended in 0 25 N-HCl at room temperature, and the suspension centrifuged to remove large insoluble debris. The supernatant suspension was then dialysed until free of Cl⁻ ions and centrifuged in a Model L Spinco ultracentrifuge at 24 000 g (av.) for 1 hr. The clear supernatant was then freeze-dried (yield 2·0 g.) and examined for its *N*-terminal groups (preparation H19).

Reaction with 1-fluoro-2 4-dinitrobenzene. A variety of conditions of coupling with 1-fluoro-2,4-dinitrobenzene (FDNB) have been tried, including an ethanol-bicarbonate medium (Sanger, 1945). In general, higher yields of total end groups were obtained in a guanidine hydrochloride-potassium bicarbonate medium, the purpose of which was to break up aggregates A typical preparation is given below. The histone hydrochloride was dissolved in 6 M-guanidine hydrochloride to give a concentration of 10-20 mg./ml. Solid KHCO₃ was added to a concentration of 10-15 mg /mL, and FDNB to 0·05-0·1 ml./mL. The mixture was shaken at about 20° for 6-24 hr. It was then acidified, diluted with 3 vol. of water and extracted once with ether, the layers being separated by centrifuging. The yellow precipitate was washed repeatedly with N-HCl and centrifuged (this did not clarify the supernatant if 0·1 N-HCl or water was used). Final traces of reagent and dinitrophenol were removed by three acetone washes and an ether wash.

The weight of dinitrophenylhistones (DNP-histones) obtained was 110-120% of the weight of histones taken. The DNP-histones were then hydrolysed, generally for 4 5 hr., in 11·1 N-HCl at 100° (Porter & Sanger, 1948), since DNP-proline was found to be an end group. The completeness of liberation of the more stable derivatives, such as DNP-valine, was checked by hydrolyses in 6 N-HCl at 110° or 120°. Hydrolyses were done in sealed tubes, with 1·5 mL of acid/ 20 mg. of DNP-histones, this being a convenient weight for one complete analysis. The hydrolysates were diluted with water and extracted four times with ether in glass-stoppered test tubes. Each ether extract was washed with 0·1 N-HCl to remove any entrained acid-soluble materials such as ε-DNP-lysine. The ether used in this work was freed from peroxide by passing through a column of activated alumina (Dasler & Bauer, 1946).

The ether-soluble materials, after sublimation of excess of dinitrophenol (Mills, 1952), were transferred in three lots of acetone to a two-dimensional chromatogram on no. 7 Whatman filter paper buffered with 0 05 M-phthalate buffer (pH 6). This was then run in the dark for 30-40 hr. with 2-methylbutan-2-ol saturated with the phthalate buffer ....

....

in the dark and the spots eluted in water and measured as described by Levy (1954) and Fraenkel-Conrat, Harris & Levy (1955). An equally satisfactory solvent was butan 2-ol saturated with the phthalate buffer, which required only 15-20 hr. to run. The phosphate was as above for the second run (see Fig 1). These alcoholic solvents were found to be more convenient than the 'toluene-ammonia' solvent advocated by Fraenkel-Conrat *et al* (1955), because no time was necessary for equilibration and because the solvent did not deteriorate. The 'toluene-ammonia' solvent was also undesirable in a laboratory where ninhydrin-amino acid determinations were being carried out.

The corrections necessary for the quantitative measurement of the *N*-terminal groups have been estimated by (a) serial measurement of each end group in samples given different periods of hydrolysis and (b) recovery experiments on the free DNP-amino acids hydrolysed in the presence of
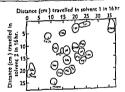
Fig. 1. Chromatography of DNP-amino acids and peptides on Whatman no. 7 paper buffered with 0·05 M-phthalate (pH 6). 0 is the starting point and both solvents were used descending the paper. Solvent I, butan-2-ol shaken with excess of 0·05 M-phthalate buffer (pH 6), upper layer used. Solvent 2, 1·5 M-sodium phosphate buffer (pH 6) and (more slowly) His, all darken in light] P²G, upper layer used. Symbols for the DNP-amino acids, etc : Asp, aspartic acid. Ala, alanine. Cy₂S₂, cystine (bis). DNA, 2 4 dinitroaniline. DNP, 2 4-dinitrophenol (spot is bleached by acid vapour). Glu, glutamic acid. Gly, glycine, His, histidine (bis). Leu, leucine and isoleucine. Lys, lysine (bis) Hypro, hydroxyproline. Pro, proline P², β-(bis) Hypro, hydroxyproline. Pro, proline P², β-chloro-α-DNP-aminovaleric acid. P², α-chloro-δ-DNP-chloro-α-DNP-aminovaleric acid. PG, prolylglycine [Hypro, Pro, PG aminovaleric acid. PG, prolylglycine [Hypro, Pro, PG here]. P²G, probably α-chloro-δ-DNP-aminovaleryl-α-DNP-aminovalerylglycine (DNP-methionine also runs here). P²G, probably α-chloro-δ-DNP-aminovaleryl-glycine. Phe, phenylalanine. Ser, serine [ε-DNP-lysine also runs here]. Thr, threonine. Tyr, tyrosine (bis). Val, valine. With 2-methylbutan-2-ol-phthalate buffer as solvent 1, the distribution of the derivatives is very similar, though the gap between the glutamic acid and serine is reduced.

The N-Terminal Groups of Calf-Thymus Histones

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It is well established that the histones of calf-thymus nucleoprotein consist of a mixture of proteins, and partial fractionations of them have been achieved (e.g. Stedman & Stedman, 1951; Davison & Butler, 1954; Daly & Mirsky, 1955; Crampton, Moore & Stein, 1955). As a continuation of the work in this Laboratory, the study of the N-terminal groups of the mixed histones from calf-thymus glands was undertaken by the fluorodinitrobenzene method (Sanger, 1945). It was expected that the results would be helpful in controlling the fractionation of the histones. While this work was in progress a dissertation on this topic appeared (Haley, 1955) and the work described in it was

later preparations of histones such as H 16 and H 17, even with the guanidine medium, the serine end groups have been as low as 5% and the lysine values down to 1% of the total.

An examination was also made of the fractions of the histones prepared by (a) extraction of the original nucleoprotein with citric acid (Davison & Butler, 1954) and (b) precipitation of the histones by sodium chloride–ammonia (Davison & Shooter, 1956). H 7/1 and H 7/2 are subfractions of the histones extracted by citric acid, which were obtained by chromatography on carboxymethyl-cellulose by the method of Davison & Shooter (1956). H 18A and H 18B are the sodium chloride–ammonia precipitate and supernatant products respectively derived from H 18, a mixture of histone preparations H 16 and H 17. The results are given in Table 2, together with those obtained from the proteins extracted from a thymus-gland brei by 0·25 N-HCl (sample H 19). This material includes much protein which is not histone. It can be seen that these various fractions gave results which differed markedly from the original histones described above.

Fractions H 7/1 and H 7/2 account for most of the histones extracted by citric acid (which are only

approximately 2% of the total histones in the nucleoprotein), and these fractions also account for most of the valine end groups found in the whole unfractionated histones. In another preparation of the citric acid-extracted histones, the proportions of the end groups in a 6 N-HCl hydrolysate (12 hr. at 110°) were: valine 39%, alanine 19%, lysine 15%, others 27%. DNP-proline and its products were completely destroyed under these conditions

Three samples of DNP-histones (H 1) have been hydrolysed under more vigorous conditions to liberate any valine which might be present as DNP-valyl-peptides (such as the DNP-valyl-ε-DNP-lysine reported by Luck et al. 1956) Some valine peptides are notoriously stable to acid hydrolysis (Synge, 1945; Porter & Sanger, 1948). However, hydrolysis in 6 N-HCl at 108° for 8 hr., at 110° for 48 hr. or at 120° for 24 hr., though increasing the . . · . . ˙ ,. · . . . . . /· · /· · · · ˙ · · · and . . .. ·. . .. " . `

valyl-ε-DNP-lysine was seen on any of these chromatograms, and only after very short hydro-lyses, e.g. 1 hr. at 100° in 11 N-HCl, did any spots other than those in the positions of the known DNP-amino acids appear. Stable peptides were also

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Table 1. *N-Terminal groups of unfractionated histones*

The proportions of *N*-terminal groups are expressed as percentages of the total end groups found.

	In guanidine hydrochloride–bicarbonate medium*			In ethanol-bicarbonate medium†
	H 1	H 16	H 17	H 1
Histone preparation				
N-Terminal amino acid				
Proline	33	45	48	40
Alanine	26	40	36	29
Serine	20	5	5	6
Lysine	7	1	2	11
Others‡	14	9	9	14
Wt. of histone/1 mole of end groups	22 500	29 000	29 000	28 000

* See Methods section.
† Ethanol 0 65 ml /ml., potassium bicarbonate 9 mg /mL, FDNB 0 03 ml./ml.
‡ Leucine (or isoleucine), valine, aspartic and glutamic acids, glycine and threonine.

Table 2. *N-Terminal groups of various histone fractions*

The proportions of the *N*-terminal groups are expressed as percentages of the total end groups found. All were coupled with FDNB in the guanidine–bicarbonate medium.

Histone fraction (for origin, see text) ...	H 7/1	H 7/2	H 18/A	H 18/B	H 19
N-Terminal amino acid					
Proline	0	39	16	66	35
Alanine	21	27	73	8	27
Serine	7	4	3 5	4	1
Lysine	16	6	2	10	3
Leucine(s)	9	5	Trace	Trace	6*
Valine	40	5	Trace	Trace	6*
Others	7	14	5 5	12	22
Wt. of histone/1 mole of end groups	62 000	14 000	27 000	27 000	45 000

* From samples hydrolysed in 6 N-HCl for 22 hr. at 110°.

value but required correction for the manipulative losses and was subject to the uncertainty that the rate of destruction of the DNP derivative in peptide form may have been different from the rate after its liberation. In (b), comparison of samples before and after hydrolysis indicated the manipulative losses in this method. These were 10–20%, depending on the derivative, but an average value of 20% loss has been adopted for all the derivatives encountered here. Much greater percentage losses occur where the amount of a given DNP-amino acid being handled is below 0·02 μmole.

The overall recoveries used in calculating the yields of end groups were: DNP-alanine 70%, bis-DNP-lysine 69%, DNP-valine and DNP-leucines 76%, DNP-aspartic acid

the recovery of small amounts of DNP-proline and relatively large amounts of its two degradation products, δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid (Scanes & Tozer, 1956). Trial degradations of DNP-prolyl-

proline the maximum yields of δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid were obtained after 0·75 and 1·5 hr. respectively in 11 N-HCl at 100°, whereas for DNP-prolylglycine the times were 3 and 5·5 hr. respectively, periods much closer to those observed in the hydrolysis of DNP-histones, in which the maximum yields of δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid were reached after 2·5 and 6 hr. respectively.

In 4·5 hr. hydrolysis the total recovery of DNP-proline and δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid from DNP-prolylglycine was 33%, and this value has been adopted as the value to be used with the DNP-histones. It is of interest that in the degradation of DNP-prolylglycine up to 2 or 3 hr. duration, spots due to δ-chloro-α-DNP-amino- and probably α-chloro-δ-DNP-amino-valerylglycine appear on the chromatograms as well as those of DNP-proline, 2·4-dinitrophenol and unchanged DNP-peptide. The positions of these substances as well as most of the common DNP-amino acids on the chromatograms are shown in Fig. 1.

The amount of DNP-proline itself on the chromatograms from DNP-histones is so small that it is unnecessary in the calculation of the yield of N-terminal proline to allow for the difference in molecular extinction coefficient between it and δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid.

The acid-soluble part of the hydrolysate of DNP-histones was evaporated to dryness and run on Whatman no. 1 paper in butanol-acetic acid–water (5·1:4, by vol.) The ε-ENP-lysine formed a wide band which may also have contained ε-DNP-lysine ... DNP ... After eluting the

as bis-DNP-lysine. No α-DNP-histidine or DNP-arginine could be detected on chromatograms of the acid-soluble remainder and no bis-DNP-histidine appeared either in the ether- or acid-soluble products.

The other part of the acid-soluble material, running more ... colourless, ... band after ow-running ... recoupled with FDNB. This was done to estimate (as bis-DNP-lysine) the proportion of free lysine in the hydrolysates of the DNP-histones, since if the original reaction with histones was incomplete free lysine would appear in the hydrolysate (assuming that lysine ε-amino groups are not blocked by some steric effect or bonding). In three hydrolysates no ... 6 N HCl hydrolysate

labelled histones and the Ehrlich test on chromatograms of barium hydroxide hydrolysates were both so weak that neither cystine nor tryptophan could contribute significantly to the end groups. The published analyses of histones show that these two acids are virtually absent (Davison & Butler, 1954; Crampton et al 1955). The ether, acid and acetone washings of DNP-histones obtained during their preparation have been examined chromatographically. Only ... trace of DNP amino acids were detected

according to the amino acid analysis published by Crampton et al (1955).

RESULTS

The N-terminal groups found in the unfractionated histones were. proline and alanine, with smaller amounts of serine, lysine, leucine or isoleucine, valine, aspartic and glutamic acids, glycine and threonine. The proportions of the end groups (expressed as percentages of the total end groups) and the total quantities obtained in the guanidine-bicarbonate and ethanol–bicarbonate media are given in Table 1. By comparing the results from histones H 1 in each reaction medium, it can be seen from the weight of histone/mole of end group that the total yield is greater in the material prepared in guanidine–bicarbonate. The duration of coupling beyond about 6 hr. had little effect on the yield of end groups

The values for serine and lysine end groups were found to vary considerably. It is thought that some of the earlier high values for serine were due to the omission of the acid washing of the ether-soluble end groups, which would result in traces of ε-DNP-lysine being measured as DNP-serine, since it runs in exactly the same position on the two-dimensional chromatograms. Nevertheless, the average yield (moles/g. of histone) of serine end groups from histone preparation H 1 was nearly four times as high when guanidine was present in the coupling medium (see Table 1), so that it would appear that in this case the ethanol–bicarbonate medium fails to allow 1-fluoro-2.4-dinitrobenzene to react completely with serine end groups. However, in the

REFERENCES

Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1955). *Nature, Lond.*, 176, 1042.

Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* 48, 126.

Crampton, C. F., Moore, S. & Stein, W. H. (1955) *J. biol. Chem.* 215, 787.

Daly, M. M. & Mirsky, A. E. (1955). *J. gen. Physiol.* 38, 405.

Dasler, W. & Bauer, C. D. (1916). *Industr. Engng Chem. (Anal.)*, 18, 52.

Davison, P. F. & Butler, J. A. V. (1954). *Biochim. biophys. Acta*, 15, 439.

Davison, P. F., James, D. W. F., Shooter, K. V. & Butler, J. A. V. (1954). *Biochim. biophys. Acta*, 15, 415.

Davison, P. F. & Shooter, K. V. (1956). *Bull. Soc. chim. Belg.* 65, 85.

Elliott, D. F. & Peart, W. S. (1957). *Biochem. J.* 65, 247.

Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955) In *Methods of Biochemical Analysis*, vol. 2, p 363. Ed. by Glick, D. New York: Interscience Publishers Inc.

Haley, M. I. (1955). *Diss. Abstr.* 15, 962.

Levy, A. L. (1954). *Nature, Lond.*, 174, 126.

Levy, A. L., Geschwind, I. I. & Li, C. H. (1955) *J. biol. Chem.* 213, 187.

Luck, J. M., Cook, H. A., Eldredge, N. T., Haley, M. I., Kupke, D. W. & Rasmussen, P. S. (1956) *Arch Biochem.* 65, 449.

Mills, G. L (1952). *Biochem. J.* 50, 707.

Porter, R. R. & Sanger, F. (1948). *Biochem. J.* 42, 287.

Sanger, F. (1945). *Biochem. J.* 39, 507.

Scanes, F. S. & Tozer, B. T. (1956). *Biochem. J.* 63, 282.

Stedman, E. & Stedman, E. (1951). *Phil. Trans* 235, 565

Synge, R. L. M. (1945) *Biochem. J.* 39, 351.

Thompson, E. O P. (1953). *Biochim. biophys Acta*, 10, 633

sought in the ethyl acetate extracts of the acid-soluble part of many of the hydrolysates of DNP-histones, but without success. Under the usual conditions of hydrolysis (4·5 hr. at 100° in 11 N-HCl) only one spot appeared on chromatograms of such extracts, in the same position as DNP-serine. However, this spot was ninhydrin-positive and proved to be ε-DNP-lysine.

DISCUSSION

The *N*-terminal groups of the thymus histones found above indicate the presence of two major types of peptide chain and about eight other types present in minor or trace amounts. The average chain weight is about 25 000. This value and the nature of the chief end groups are, with the exception of alanine, at variance with the results of Haley (1955) and Luck *et al.* (1956), which were obtained with histones prepared by extraction with sodium chloride and hydrochloric acid of thymus-cell nuclei. In whole-thymus histones these authors found that valine accounted for 53 % of the *N*-terminal residues, leucine for 21 %, alanine for 18 % and glycine for 8 %. The value of 1 g. mol. of

except on the assumption that degradation of the histones occurred in the preparative method used by Luck *et al.* (1956). Although the histones are known to aggregate in alkaline solution (Davison *et al.* 1954), the reaction with 1-fluoro-2:4-dinitro-benzene is thought to have been complete since virtually no free lysine could be detected in the hydrolysates of DNP-histones. The hydrolyses are thought to have liberated most of the end groups since more severe conditions did not raise the values of terminal DNP-valine and DNP-leucine (which can give very stable DNP-peptides) to values anywhere near those of DNP-proline or DNP-alanine. The corrections for hydrolytic and manipulative losses are thought to have been adequate, though in some work with DNP-proteins, e.g. with hypertensin (Elliott & Peart, 1957) and with corticotrophin (Levy, Geschwind & Li, 1955), the losses of terminal DNP-amino acids were greater than expected. Finally, the washings from the preparations of the DNP-histones have revealed no loss of terminal groups such as Thompson (1953) encountered in the study of carboxypeptidase.

Experiments made with the object of causing slight degradation of the histones, by keeping a solution at pH 7 for 2 days or in 0·1 N-sodium hydroxide for a day, or by heating the aqueous solution at 100° for an hour, caused only very slight increases in some of the end groups. Another experiment wherein all the acid-soluble protein

(not merely the histones) from thymus glands was coupled, after dialysis, with 1-fluoro-2:4-dinitro-benzene showed that the amounts of valine and leucine end groups (each was 6 % of the total) were small compared with the proline and alanine end groups, which were 35 and 27 % of the total end groups respectively.

The sodium chloride–ammonia fractionation of the histones has been shown to precipitate the histones which are relatively richer in arginine and migrate faster on electrophoresis, and to leave in the solution the histones which are relatively richer in lysine and which migrate more slowly on electrophoresis (Davison & Shooter, 1956). Table 2 shows further that most of the arginine-rich histones (H 18A) have alanine *N*-terminal groups and most of the lysine-rich ones (H 18B) have proline *N*-terminal groups. The small amounts of histones with other end groups fall into both of these main classes.

The origin of the differences in yield of serine and lysine end groups in different preparations of the mixed histones has not been established. In the later preparations both of these acids formed only a minor proportion of the *N*-terminal groups. There may be variations in the proportions of the different histones obtained from different animals or breeds of cattle. Allfrey, Mirsky & Osawa (1955) have noted the variability in properties of thymus nuclei prepared at different times of the year. Moreover, in the preparative stages involving washings and dialyses near neutrality there is the possibility of enzyme action causing slight degradation of peptide chains.

SUMMARY

1. The chief *N*-terminal groups of the histones extracted from calf-thymus glands are proline and alanine, with smaller amounts of lysine and serine, and still less of six other amino acids.

2. The proportions of the serine and lysine end groups have declined markedly in later preparations of the histones.

3. The quantities of the end groups gave an average weight of about 25 000/g. mol. of end groups.

4. In a fractionation of the histones, those precipitated by sodium chloride–ammonia (the arginine-rich histones) mostly have alanine *N*-terminal groups, whereas those remaining in solution in sodium chloride–ammonia (the lysine-rich histones) mostly have proline end groups.

In the case of flexible synthetic polyelectrolytes, it was originally found by Fuoss & Strauss (1948) that the reduced viscosity increases steadily with decreasing concentration, which was attributed to an expansion of the particle as the concentration is decreased. However, in certain cases it has been observed that on continuing the measurements to very low concentrations, a maximum of the reduced viscosity is reached and at lower concentrations it falls again to smaller values (Conway & Butler 1953 b; Eisenberg & Pouyet 1954; Conway 1955). It would thus appear that the behaviour observed in any case will be determined by the relative effects of changes of concentration on particle interactions, and on the intramolecular factors determining its extension. The former may be expected to increase rapidly with the size of the particle and may, therefore, not become a predominating effect in dilute solutions except with extremely large and elongated particles. Thus Fujita & Homma (1954) using carboxymethyl cellulose, found that maxima in the curves of reduced viscosity against concentration for fractions of low molecular weight, give place to flat plateaux when the molecular weight increases.

It is evidently desirable that a greater variety of polyelectrolytes should be studied in this way. Those which are polymers of weak acids, such as methacrylic acid, are susceptible to effects which may cause an apparent decrease of the reduced viscosity in water at high dilutions, e.g. (a) hydrolysis of the polyelectrolyte salt in water, as

$$RNa_x + H_2O \rightleftharpoons RNa_{x-1} + NaOH, \quad \text{etc.,}$$

and (b) conversion of the salt into the corresponding acid owing to the absorption of carbon dioxide from the atmosphere, which is difficult to avoid entirely, by

$$RNa_x + H_2CO_3 \rightleftharpoons RNa_{x-1} + NaHCO_3.$$

These reactions were considered by Conway & Butler (1953 b) and Conway (1955) in the investigation of polymethacrylic acid, and various tests were given which indicated them to be unimportant under the experimental conditions employed. A further test can be made in which such changes of the acid/salt ratio are avoided by using a salt of a strong polyacid or polybase. In the case of polyvinyl (N-n-butyl) pyridinium bromide, which probably belongs to this class, maxima in the curves of reduced viscosity against concentration have been observed by Eisenberg & Pouyet (1954) and by Conway (1955). As a further test we have studied the sodium salt of polystyrene sulphonic acid, manufactured by the Monsanto Chemical Co. and marketed under the name of Lustrex. The behaviour of this material has been examined both as supplied and after some degree of fractionation, the object of which was to produce variations of the average particle size. The effect of added salts on these materials was also examined, and in order to obtain an estimate of the degree of dissociation of the polyelectrolyte, measurements were made of electrical conductivity of the solutions in water.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Characteristics of sulphonated polystyrene (Lustrex 770)*</div>

The sodium polystyrene sulphonate used was a sample of Lustrex 770 supplied by the Monsanto Chemical Co. As received it contained 8% moisture. Analytical data obtained by Miss J. Cuckney and staff of the Microanalytical Laboratories of

The viscous behaviour of dilute solutions of a strong polyelectrolyte (polystyrene sulphonate)

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Few investigations of the behaviour of polyelectrolyte solutions have been carried to sufficiently low concentrations to ascertain the limiting value of the reduced specific viscosity at infinite . . . .

investigation has been made into the viscous behaviour of polymers of the strong acid styrene sulphonic acid and its sodium salt in dilute solutions. These materials show a maximum reduced specific viscosity at a concentration of ca. 0·002 %, which appears to be real, although its magnitude is greatly influenced by the purity of the water and small concentrations of ions have a very marked effect. It thus cannot be concluded with certainty that the observed increase of the reduced specific viscosity with concentration at low concentrations is due to interaction effects.

The molecular weights of the fractions used were determined from their mean sedimentation constants and intrinsic viscosities using the equation of Flory & Mandelkern (1952). From these molecular weights, mean end-to-end lengths of the particles under different conditions *were derived by the relation of Flory & Fox* (1951). It was found that at the maximum of the viscosity curve, the extension of the particle in water is about half of the total contour length and is sufficient to permit of particle interaction at these concentrations. The length is greatly diminished by increase of the ionic strength, and even if interaction occurs at low concentrations, it ceases to be the dominant factor when the concentration is greater than 0·003 % in most cases.

Measurements were also made of the electrical conductance of the polymer in the salt and acid forms. In the latter it was found that although the degree of dissociation is low, the equivalent conductance is nearly independent of the concentration. This would appear to mean that the dissociation of the hydrogen ions is determined by the micro-environment of the acid groups within the particles and is scarcely influenced by the concentration of polymer particles in the solution.

INTRODUCTION

From the intrinsic viscosity of high polymers, information about the shape and size of the isolated particles can be deduced and a study of the effects of changes of concentration and of added ions in solution may be expected to indicate the changes of shape and size and particle interaction brought about by these variables.

In the case of sodium deoxyribonucleate (*DNA*) solutions in water, it was shown by Conway & Butler (1953*a*) that the reduced viscosity, $[\eta/\eta_0 - 1]/c$, when measured at very low shear rates in the absence of added salts, increases continuously with increasing concentration. Since added salts have little effect on the intrinsic viscosity of these solutions (Pouyet 1952; Conway & Butler 1954), it has been concluded that in this case the shape of the particle is to a large extent independent of the concentration of added electrolyte, and that the increase of the reduced viscosity with concentration of the polymer, is due to increasing particle interactions. The nature of these interactions is still uncertain, although the great effect of small additions of ions indicates that they have an electrostatic origin (Butler, Conway & James 1954)

first against distilled water and then against conductivity water for 24 h. The solution was evaporated gently to dryness at 70° C, the solid being finally dried at 110° C.

Preparation of polystyrene sulphonic acid

It was hoped that by the addition of a large excess of strong acid, most of the Lustrex would be precipitated from an aqueous solution in the acid form (Mock & Marshall 1955). It was found, however, that when concentrated hydrochloric acid, fortified with some sulphuric acid, was employed, only 37 % by weight of the polymer was precipitated and only 80 % of this was in the acid form. Dialysis of the precipitate, after solution in water, led to chloride- and sulphate-free solutions, and the final dialysis was conducted against conductivity water. Evaporation of the solution was again carried out at 70° C. From the estimated molecular weight of the precipitated sample, $A\,2$, after neutralization (see table 1) it is evident that some degree of fractionation has unavoidably occurred.

The use of cation-exchange resins suggested itself as a convenient method of producing the free polymeric acid. Accordingly, a 0·1 % solution of Lustrex was percolated down a column of Zeo-carb 225 resin, in the hydrogen form. Between 93 and 98 % of the polymer was recovered in different runs ($A\,3$), indicating that little fractionation had taken place. The extent of ion exchange was 75 %. The effluent was dialyzed against conductivity water before evaporating to dryness or freeze-drying. A disadvantage of this method is the low concentration necessary to obtain a reasonable percolation rate.

Stability of the polyelectrolyte

(i) *Solid*

The sodium polystyrene sulphonate is very hygroscopic, and must be dried at 110°C for at least 2 h before solutions can be made up by weight. No decomposition of the solid was observed up to 130° C. In practice the concentration of the stock solution was measured by evaporation of aliquots to constant weight.

When the acid form of the polymer was evaporated to dryness it rapidly became discoloured at 110° C. Prolongation of the heating at this temperature beyond 15 min led to the formation of an insoluble product. This sensitiveness of the sulphonic acid to heat has been reported by Demagistri (1950). The production of an insoluble product when the temperature is raised may be the result of chemical reaction leading to cross-linking through sulphonic acid groups. Signer & Demagistri (1950) report that sulphonation of polystyrene by oleum leads to products which swell and do not dissolve in water, due to sulphone formation. In our case, this side reaction could be avoided by freeze-drying of the solutions of polyacid.

(ii) *Solutions*

The solutions of polyelectrolyte were made up in conductivity water unless otherwise specified. Twice-distilled water was allowed to percolate through a bed of B.D.H. mixed-bed resin (m.b.1.) using guard tubes of soda-lime to exclude carbon dioxide. The conductivity of the eluate was $0·5 \times 10^{-6}\ \Omega^{-1}\ cm^{-1}$.

Imperial College, London, on the dried material were reported as C, 41·7; H, 3·4; S, 14·7; Na 12·0 %; corresponding to the empirical formula $C_8H_{7\cdot9}S_{1\cdot0}Na_{1\cdot21}$. If the material is consistently mono-sulphonated this indicates the presence of about 6 % of sodium sulphate. The presence of this amount of salt was not considered to be a serious matter at low concentrations of the polymer, since at 0·001 % polymer it would lead to a concentration of counter-ions from this source of about 1×10^{-5} g ion l.$^{-1}$. Dialysis of the solutions leads to a small increase of the specific viscosity at all concentrations, but this is partly due to the loss of polymer particles of low molecular weight. However, dialysis did not change the character of the viscosity curves. The fractionated materials described below were all dialyzed before use. The quality of the water used was of considerable importance in determining the shape of the viscosity curve at low concentration.

The partial specific volume of the polymer was determined by measurement of the density of solutions between 0·2 and 0·8 % in 0·2 M sodium chloride using a pyknometer at 20·0° C. The value obtained was 0·697 ± 0·013 ml./g.

The ultra-violet absorption spectrum of a 0·1 % aqueous solution was measured by Dr P. D. Lawley. By taking the equivalent weight of the polyelectrolyte as 206, corresponding to $C_8H_7SO_3Na$, the equivalent extinction coefficients at the maxima and minima were calculated:

λ (mμ)	249	255·5	258	261·5	267	272
ϵ	262	321	304	361	282	196
	min.	max.	min.	max.		

Fractionation of sodium polystyrene sulphonate

Mock & Marshall (1955) succeeded in fractionating this material to give samples possessing an average molecular weight of 8×10^6, by precipitation of fractions from a 1 % aqueous solution of the polyelectrolyte with 9 M sodium iodide. The starting material used by these authors had a weight-average molecular weight of 529 000. It was found when attempting to repeat this process with the Lustrex 770 sample that the addition to a 1 % solution of sodium iodide to 4 M caused the precipitation of the first 5 % by weight. On adding small quantities of sodium iodide to the supernatant further fractions could be obtained. The precipitates adhered to the stirrer and could be separated readily from the solution. They were dissolved in the minimum volume of water, precipitated with absolute alcohol, and then dried to a pale yellow powder. Dilute aqueous solutions were made as required and dialyzed before use.

It was difficult to remove the yellow coloration from the fraction so prepared and accordingly an alternative method of fractionation was attempted. Concentrated hydrochloric acid was added to a 1 % aqueous solution of Lustrex and precipitation commenced at an acid strength of 6 N. Care was again exercised to ensure that only 10 to 15 % of the polymer was precipitated, and the fraction obtained (A 1) was very similar to that which resulted from the use of sodium iodide. The acid-precipitated material was dissolved in water and reprecipitated with hydrochloric acid. Finally, it was redissolved in water, neutralized with sodium hydroxide, and dialyzed

to avoid errors arising from adsorption of the polyelectrolyte upon the vessel and the electrodes. Each measurement of cell resistance was made after two or three complete fillings of the cell, with intervals of 10–15 min between each. On standing for this period, the cell resistance in the first filling, and occasionally in the second, increased slowly. It fell again when the cell was re-filled with fresh solution, but after the third filling this behaviour ceased. The adsorption effect was marked in the case of solutions of the polyacid, but was much less so with the salt. Since the sample of polyacid still contained up to 33 % of sodium salt, the contribution of the latter had to be allowed for when calculating the equivalent conductivity of the acid form.

RESULTS

Viscosity measurements

The viscosity measurements are presented in terms of the reduced viscosities in decilitres/gram plotted against the concentration of polyelectrolyte in grams/decilitre or percentage weight.
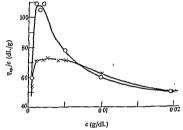
FIGURE 1. Reduced viscosity plotted against concentration for aqueous solutions of sodium polystyrene sulphonate. O, Conductivity water; ×, twice-distilled water.

(i) Sodium polystyrene sulphonate

The curves of the unfractionated material are shown in figure 1. When twice glass-distilled water was used, a flat maximum was observed in the curve between 0·004 and 0·01 %. When the solutions were made up in conductivity water, the maximum was sharp and occurred at a concentration of about 0·001 %. Little difference between distilled and conductivity water was observed at a higher concentration, but if dialysis had been carried out the whole curve was moved to higher values of reduced viscosity. Since there is very little change of the pH during dialysis, it is possible that some fractionation of the polymer occurs by the diffusion of low molecular weight material out of the dialysis bag. The rise may also result from the removal of traces of ionic impurities. In view of the uncertainties arising from dialysis, this process was not used except after neutralization of fractionated samples, as has already been indicated, and after column percolation.

The viscosity of dilute polystyrene sulphonate solutions in glass vessels decreased slowly on standing. This decrease may be due to (a) the leaching of sodium ions from the glass; (b) the absorption of carbon dioxide from air with the resulting formation of bicarbonate ions. It has been shown (Beattie 1953) that the leaching of fresh glass surfaces proceeds at a moderate rate. The deterioration was greatly reduced by storing all solutions in polyethylene vessels at a concentration of more than 0·02 %. Under these conditions only slight changes of viscosity occurred during several weeks. In view of this stability it appears to be unlikely that the absorption of carbon dioxide is an important factor. The polyethylene vessels were kept closed, but rigid precautions to exclude carbon dioxide were not made, as the viscosity measurements necessarily involved some exposure to the atmosphere.

The following procedure for handling the solutions was adopted as standard and gave reproducible results. The stock solutions from which all dilutions were made immediately prior to their investigation, and having a concentration of 0·02 % or greater, were stored in a polyethylene vessel. The concentration of the solutions was measured by evaporation of 10 ml. portions to constant weight. The Couette cylinders were rinsed twice with the solution under test. Some of the solution was then allowed to stand in the apparatus for 15 min to eliminate adsorption errors. This sample was then rejected and the apparatus was filled with a further sample, previously warmed to 25°C, and measurements were made at once. In the case of the low viscosity, unfractionated samples, this 'conditioning' procedure made little or no difference, but with the higher molecular weight samples it was important.

The viscosimeter

Measurements of viscosity were made using a Couette-type of viscosimeter similar to that described by Ogston & Stanier (1953). The cylinders were of stainless steel; the inner one having a diameter of 1·25 cm and the outer one a diameter of 1·50 cm. The length of the inner cylinder was 8·9 cm and it was suspended by a beryllium-copper wire of diameter 0·004 in. and length 30 cm, and also carried a concave mirror of focal length 50 cm. The outer cylinder, which was thermostatically controlled at 25 ± 0·05° C by rapidly circulating water, was rotated through a system of belts and pulleys by an induction motor geared to give an output of 10 rev/min. The shear-rates to which the liquid in the annulus was subjected ranged from 1 to 30 s⁻¹.

The viscosity was independent of the rate of shear within these limits, except with the fractions precipitated by sodium iodide and hydrochloric acid, for which the viscosity decreased slightly over this range with increasing rates of shear. In these cases the specific viscosity at zero shear rate was obtained by extrapolation, the correction applied being only a few parts in a hundred.

Conductivity measurements

These were made on a modified Jones–Josephs bridge (Jones & Josephs 1928) by the kindness of Dr F. J. F. Dippy, in the Department of Chemistry, Chelsea Polytechnic. The cell used was of large capacity (250 ml) and had a constant of 0·264. The electrodes were of platinized platinum and sealed into the glass supports in such a way that only one surface of each was presented to the solution. Care was exercised
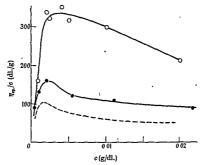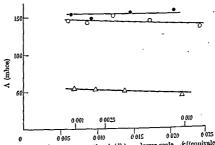
FIGURE 4. Reduced viscosity plotted against concentration for solutions of polystyrene sulphonic acid prepared by HCl precipitation. O, Free acid in conductivity water; ●, 'neutralized acid' in conductivity water; - - -, original unfractionated sodium salt (see figure 1).

FIGURE 5. Equivalent conductance as a function of the square root of concentration. ●, Acid form of polyelectrolyte at 25° C; O, acid form of polyelectrolyte at 20° C (different preparation); △, sodium form of polyelectrolyte at 25° C.

Sedimentation coefficients and molecular weights

Sedimentation experiments were performed on solutions of sodium polystyrene sulphonate in 0·2 M sodium chloride using a Spinco analytical ultra-centrifuge. Measurements with solutions of the original unfractionated material were made at concentrations of 0·74 and 0·37 % using the Philpot–Svensson optical system, and at 0·074 and 0·037 % using the ultra-violet absorption system. The weight-average sedimentation constant at infinite dilution was obtained by extrapolating the

Figures 2, 3 show the reduced viscosities of the polystyrene sulphonate solutions in the presence of added sodium chloride. The effect was especially marked in the case of samples of fractionated polymer (figure 3).

FIGURE 2

FIGURE 3

c (g/dl.)
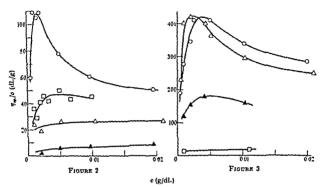
FIGURE 2. Reduced viscosity plotted against concentration for aqueous and saline solutions of sodium polystyrene sulphonate. O, Conductivity water; □, 5×10^{-4} M NaCl; △, 7×10^{-4} M NaCl; ▲, 8×10^{-5} M NaCl.

FIGURE 3. Reduced viscosity plotted against concentration for solutions of fractionated sodium polystyrene sulphonate. Fractions obtained: O, by HCl precipitation, in water; △, by NaI precipitation, in water; ▲, by NaI precipitation, in 4×10^{-4} M NaCl; □, by NaI precipitation, in 4×10^{-4} M NaCl.

(ii) *Polystyrene sulphonic acid*

The viscosities of polystyrene sulphonic acid (figure 4) are higher than those of the sodium salt, from which it was obtained by precipitation. However, part of the increase is due to fractionation in the precipitation, since on neutralizing the polymer acid with sodium hydroxide, the viscosities drop, but do not return entirely to the values obtained for the original salt.

Conductivity measurements

These measurements were spread over the concentration region where the specific viscosity was changing most rapidly with concentration. It is obvious from figure 5 that the equivalent conductivities of both polyacid and salt are only slightly dependent on the concentration. The values for the polyacid are ca. 44 % of the value of the hydrogen ion conductance alone. Without a knowledge of the mobilities of the polymer particles, the degrees of dissociation cannot be stated, but it is evident that while the dissociation of the acid form is far from complete, the dissociation of the sodium salt is comparatively high.

Similar calculations using the values of the reduced viscosities of the neutralized solutions at the maxima of the curves in water give the maximum end-to-end distance R', as shown in table 2. R_∞, the contour length of the polymer chain derived from the molecular weight taking the repeat distance to be 2.6 Å is also given for comparison.

TABLE 1. INTRINSIC VISCOSITY AND SEDIMENTATION DATA ON POLYSTYRENE SULPHONATE (LUSTREX 770) AND FRACTIONS DERIVED FROM IT

fraction	$[\eta]$ (dl./g)	$10^{13} S_0$	$10^{-4} \bar{M}$	R_0 (Å)
original material	1·4	9·0 、	6·8	770
sodium iodide fraction	2·5	10 6	11·5	1110
hydrochloric acid fraction A 1*	1·96	11·5	11·5	1030
acid form A 2*	2·0	9 05	8·1	920
acid form A 3*	1·6	8·6	6·7	800

* Sedimentation coefficients and viscosities were measured after neutralization of these fractions.

TABLE 2. COMPARISON BETWEEN CALCULATED MOLECULAR DIMENSIONS

fraction	(η_{sp}/c_{max}) (dl./g)	R' (Å)	R_∞ (Å)	R_0 (Å)
original material	110	3290	8600	770
sodium iodide fraction	420	6120	14500	1110
hydrochloric acid fraction A 1	420	6120	14500	1030
acid form A 2	160	3950	10200	920
acid form A 3	100	3290	8600	800

DISCUSSION

Although maxima in the curves of reduced viscosity against concentration have been observed in all cases of the solutions in water which were examined, it cannot be regarded as proved that the decrease in the reduced viscosity at concentrations below the maximum is due to a diminution of the interaction between particles. The result of a greater degree of purification of the water used has been to displace the maximum towards lower concentrations. It then occurs at such low concentrations that it is possible that the values of the reduced viscosity are influenced either by the residual ions present in the water or by counter ions from the polyelectrolyte itself. The viscosity of the polystyrene sulphonate solutions has been shown to be extremely sensitive to the presence of added ions, even a concentration of 4×10^{-5} M sodium chloride being sufficient to modify markedly the curve of η_{sp}/c against c. The substance itself is a strong electrolyte and gives rise to appreciable concentrations of ions. Assuming complete dissociation, the sodium ion concentration derived from the polyelectrolyte in a 0·001 % solution will be of the order of 5×10^{-5} M. It has been shown by conductivity measurements that the degree of dissociation, though not complete, is quite high, so that the sodium ion concentration arising from the polyelectrolyte may be of considerable importance. At concentrations below 0·001 % we are, moreover, in a region in which the presence of residual ions in the solvent is of great importance.

relation between S_0 and c. Measurements on solutions of the fractionated material were made at concentrations of 0·07, 0·04 and 0·01 % using the Philpot–Svensson system at the highest concentration and the absorption system for the lower concentrations. However, the sedimentation coefficient measured from the maximum of the schlieren peak at 0·07 % did not appear to correspond very closely with the average measured using the absorption system. An approximate value for the weight-average sedimentation constant at zero concentration, S_0, was obtained in these cases by extrapolation from the results for the two lower concentrations. The average sedimentation coefficients at 0·04 and 0·01 % did not differ by more than 0·5 svedbergs.
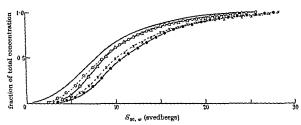
Figure 6. Integral distribution curves of sedimentation coefficients plotted against fraction of total concentration. ——, Original material (see figure 1), 0·037 %; O, acid form obtained by ion exchange and freeze dried, 0·01 %; △, acid form obtained by HCl precipitation (see figure 4), 0·01 %; ×, fraction from NaI precipitation (see figure 3), 0·01 %; ●, fraction from HCl precipitation (see figure 3), 0·01 %.

From the experiments with the absorption system, curves showing the distribution of sedimentation coefficients were calculated in the manner described by Shooter & Butler (1956). The curves observed using the lowest concentrations are compared in figure 6. Although fractionation removed some of the lower molecular weight material, the general shape of the distribution and the degree of heterogeneity are not greatly changed.

Molecular weights of the various fractions when neutralized ($\bar{M}$), have been calculated from the intrinsic viscosities $[\eta]$, determined by extrapolating η_{sp}/c, taken at zero shear, to zero concentration in 0·2 M sodium chloride, and S_0, using the equation of Flory & Mandelkern (1952):

$$\frac{S_0 [\eta]^{\frac{1}{3}}}{\bar{M}^{\frac{2}{3}}} = \frac{2 \cdot 6 \times 10^6 (1 - \bar{V} \rho)}{N \eta_0}.$$

Where $\bar{V}$ is the partial specific volume, ρ the density and η_0 the viscosity of the solvent. The values obtained are given in table 1, where R_0 is the mean end-to-end length in salt as given by the relation of Flory & Fox (1951):

$$R_0^3 = \bar{M} [\eta]/2 \cdot 1 \times 10^{21}.$$

It would appear to be necessary to suppose that the equilibrium of protons between sulphonic groups and water is independent of the concentration, at least over the range examined and that the dissociation of the —SO_3H groups is determined by their micro-environment and is largely insulated from the state of the whole solution.

The equivalent conductance of the sodium salt is about equal to that of sodium ions, and therefore, assuming that the mobility of the polymer ion is small, the degree of dissociation is high. The behaviour of the sodium salt is thus not unusual.

REFERENCES

Basu, S. & Sircar, A. K. 1954 *J. Colloid Sci.* 9, 574.
Basu, S. & Sircar, A. K. 1955 *J. Colloid Sci.* 10, 263.
Beattie, I. R. 1953 *Tran Faraday Soc.* 49, 1059.
Butler, J. A. V., Conway, B. E. & James, D. W. F. 1954 *Trans Faraday Soc.* 50, 612.
Conway, B. E. 1955 *J. Polymer Sci.* 18, 257.
Conway, B. E. & Butler, J. A. V. 1953a *J. Polymer Sci.* 11, 277.
Conway, B. E. & Butler, J. A. V. 1953b *Nature, Lond.,* 172, 153.
Conway, B. E. & Butler, J. A. V. 1954 *J. Polymer Sci.* 12, 199.
Demagistri, A. 1950 Thesis, Bern.
Eisenberg, H. & Pouyet, J. 1954 *J. Polymer Sci.* 13, 85.
Flory, P. J. & Fox, T. G. 1951 *J. Amer. Chem. Soc.* 73, 1909.
Flory, P. J. & Mandelkern, L. 1952 *J. Chem. Phys.* 20, 212.
Fujita, H. & Homma, T. 1954 *J. Colloid Sci.* 9, 591.
Fuoss, R. M. & Strauss, U. R. 1948 *J. Polymer Sci.* 3, 246, 602.
Jones, G. & Josephs, R. C. 1928 *J. Amer. Chem. Soc.* 50, 1049.
Mock, R. A. & Marshall, C. A. 1954 *J. Polymer Sci.* 13, 263.
Mock, R. A. & Marshall, C. A. 1955 *J. Polymer Sci.* 17, 591.
Mock, R. A., Marshall, C. A. & Slykhouse, T. E. 1954 *J. Phys. Chem.* 58, 498.
O. · C. & Si----- I. E. 1953 *Biochem. J.* 53, 4.
                              Paris, 234, 152.
    . .   . . . 1956 *Trans. Faraday, Soc.* 53, 734
Signer, R. & Demagistri, A. 1950 *J. Chim. phys.* 47, 704.

It may be seen from table 2. which gives the mean end-to-end length of the particles under different conditions for the various fractions, calculated from the Flory–Fox relation, that even at the observed maximum of the reduced viscosity, the extension of the particle is less than half the contour length required by the molecular weight. A very small concentration of salt reduces this markedly, and in 0·2 M sodium chloride the extension is about one-tenth of the contour length It would appear, therefore, that at the lowest concentrations studied, the particles are not fully extended, but their end-to-end extension is of the same order as that of *DNA* particles in solution, which show marked interaction in solution at a similar concentration. It is thus reasonable to expect that appreciable interaction occurs at the maximum of the curve, but if the extension of the particle is a diminishing function of the concentration, interaction will soon cease to be the predominant factor at higher concentrations.

The main cause of the difference of behaviour appears, therefore, to be the great effect of ionic strength on the extension of the polystyrene sulphonate particle as compared with *DNA*. One reason for this may well be the mutual attractions between the aromatic rings of the former, which act in an opposite direction to the repulsions of the charges carried on the macromolecule. While in *DNA* the structure itself provides rigidity, in polystyrene sulphonate a sensitive state of balance of the forces causes contraction and expansion

The difference between the reduced viscosities of the neutral and acid forms of the polyelectrolyte is of some interest (figure 4). Since conductivity measurements have indicated that the hydrogen ion is less dissociated from the polyelectrolyte ion than the sodium ion, it is not to be expected that the acid form will have a greater extension in solution. The viscosity *measurements would thus suggest that the greater value of the reduced viscosity of the acid form results from particle inter-actions produced by hydrogen bonding between sulphonic groups.* Very similar results have been reported by Basu & Sircar (1954), who found that the reduced viscosity of agar acid in water is greater than the viscosity of the sodium salt at all concentrations. This behaviour has been tentatively attributed to hydrogen bonding in the agar acid. A similar study of gum tragacanth (Basu & Sircar 1955) revealed behaviour similar to that of polymethacrylic acid, namely the acid form at all concentrations has a lower viscosity than the neutral salt. It is significant that both polystyrene sulphonic acid and gum agar acid possess sulphonic acid groups, whereas *in gum tragacanth and polymethacrylic acid, the ionizing group is carboxylic.*

The electrical conductivities of the solutions also require some comment. The equivalent conductance of the polyelectrolyte in the acid form is only about 40 % of that of the hydrogen ions, showing that dissociation is very incomplete. It is, therefore, remarkable to find that the equivalent conductance is independent of the concentration over the range examined. A similar behaviour has been found with vinyl toluene-styrene co-polymer sulphonic acid by Mock & Marshall (1954) and Mock, Marshall & Slykhouse (1954), who suggested that 'the number of protons trapped within the swollen ellipsoid probably remains invariant with changing polymer concentration', although they pointed out that this is inconsistent with the inference from viscosity that the volume of the ellipsoid changes with the concentration.

Perfusion.—The rats were killed by perfusion of ice-cold sucrose (0·25 M) or NaCl (0·85 per cent) into the aorta under pentobarbitone anaesthesia. The tissues were quickly removed and kept at 0° to 4° or at −20° if not used on the same day.

Isolation of total proteins.—Weighed samples of tissue were placed in cold 10 per cent (w/v) " Analar " trichloroacetic acid (TCA) and a suspension was prepared with the aid of a blendor of the cutting edge type (M.S.E. Ltd). After centrifugation the sediment was washed with TCA (10 per cent, w/v), then with TCA (5 per cent, w/v) followed by water and ethanol. The sediment was then extracted with boiling ethanol-chloroform (1 : 1) twice for 20 minutes and then washed twice with acetone. If no samples were required for radioactive assay at this stage the residue was washed with ethanol and then treated with TCA (5 per cent, w/v) at 90° for 20 minutes. After centrifugation the sediment was washed once with TCA (5 per cent, w/v), then with water followed by acetone. The moist residues were dried at not more than 80°.

Extraction of nucleic acids.—Samples of deoxyribonucleic acid (DNA) were prepared by washing and centrifuging homogenized spleen or thymus tissue with NaCl (0·145 M) several times. The residue was then treated with sodium dodecyl-sulphate (Jones and Marsh, 1954) and the resulting impure DNA precipitated twice with ethanol. Further deproteinization with chloroform-*n*-octanol (Sevag) usually followed if the size of the sample permitted. Ribonucleic acid and DNA were also isolated after treatment of the tissue with a two-phase system of phenol and water (Kirby, 1956a, 1956b). The nucleic acids were reprecipitated with ethanol twice. The protein fraction was isolated from the phenolic layer by the addition of ethanol and washing the precipitate with ethanol-water (3 : 1). Treatment with hot ethanol-chloroform and hot TCA then followed as described above.

Differential centrifugation.—Each gram of liver or kidney was dispersed with 5 ml of ice-cold sucrose (0·25 M) in a mechanically driven homogenizer of the Potter-Elvehjem type with a polythene pestle until almost no whole cells could be seen on microscopic inspection. The suspension was strained through 3 layers of gauze and centrifuged at 400 × g (average) for 10 minutes. The sediment was resuspended in sucrose (0·25 M) with the aid of the homogenizer, centrifuged and dispersed in ice-cold water. Citric acid (0·025 M) was carefully added to lower the pH to 6·1. After centrifugation at 300 × g for 8 minutes the loosely packed sediment was often suspended in cold water and centrifuged again. This separation of the nuclear fraction was followed microscopically. The combined cytoplasmic supernatant liquids were centrifuged at 10,000 r.p.m. (= 10,000 × g average) in a No. 21 head of the Spinco ultracentrifuge for 10 minutes. The mitochondrial fraction was resuspended in sucrose and recentrifuged. The combined supernatant liquids were centrifuged at 26,500 × g for 30 minutes to yield the microsomal and the supernatant fractions. TCA was added to all fractions which were then treated as described above.

Experiments on tissue suspensions and proteins in vitro.—Tissue was homogenized in sucrose (0·25 M) or NaCl (0·85 per cent), a freshly prepared solution of the sodium salt of PAM (see above) or a hydrolysate of PAM (see below) was added. In some experiments the tissue suspension was replaced by a solution (2·5 per cent, w/v) of plasma albumin (Armour). After a given period of time precipitating agents were added and the sediments washed twice with the same reagents and then processed as described above.

TABLE I.—*Specific Radioactivities of Walker Carcinoma and of Proteins of Several Tissues of the Rat after the Administration of PAM*

| Experiment number | Dose (mg./kg.) | Period of exposure to PAM (days) | Walker carcinoma present at | | Radioactivity (µc/g.) of | | | | | | |
| | | | Beginning | End | Tumour | | | Liver | Kidney proteins | Spleen proteins | Thymus proteins |
					Whole tissue	Proteins	Blood proteins				
1	15	2	+	+	—	0·014*	0·034	0·038	0·18	—	—
2	22·5	4	+	+	0·090	0·060	0·049	0·046	0·24	—	—
3	25	4	+	+	0·072	0·069	—	0·035	0·24	—	—
4	15	4	+	+	0·056	0·050	0·039	0·025	0·17	—	—
5	15	5	+	+	0·057	0·031	0·031	0·025	0·11	—	—
6	2·5	8	+	+	—	0·007	—	0·006	—	—	—
7	2·5	8	—	—	—	—	—	0·040	—	—	—
8	5	8	+	—	—	0·012	—	0·007	—	—	—
9	10	8	+	—	—	—	—	0·018	0·045	—	—
10	10 then 5	1	Not implanted		—	—	—	0·062	0·18	0·042†	0·078*
11	10 then 10	4 then 3	Ditto		—	—	—	0·033	0·13		

* Tissue treated with cold TCA, washed and dried with acetone.
† Tissue proteins precipitated with cold TCA, washed and lipid extracted

Tumours implanted thirteen days before intraperitoneal administration of a suspension of the compound in arachis oil.
Radioactivity of compound . 4·2 µc/mg.
Range of weight of rats 250–360 g.
Rats killed by perfusion with NaCl (0·85 per cent) under pentobarbitone anaesthesia.

almost one-fifth of the samples from the liver and nearly one-half of those from the kidney in the experiments shown in Fig. 1 to lose between 21 and 35 per cent of their specific radioactivity. When perchloric acid (PCA) was used in the same manner as TCA the specific radioactivities were about 15 per cent lower than with TCA. These results suggested that radioactive material removed by hot TCA and PCA might have been bound to the nucleic acids which would thus have possessed a greater specific radioactivity than the proteins. Samples of DNA or RNA as well as of the protein fraction of the same tissue were therefore prepared in an attempt to obtain direct evidence on this point; but in view of the amounts available they could often not be highly purified. Even if the residual protein attached to the nucleic acids possessed no radioactivity the specific radioactivity of these samples of nucleic acids would still have been less than those of the protein fractions. Hence it follows that by the above mentioned treatment with TCA or PCA of proteins and nucleoproteins of tissues not only nucleic acids but also some other radioactive material bound to proteins have been extracted.

The specific radioactivities of cell fractions of livers and kidneys from rats exposed to PAM for selected periods of time were then compared to obtain further evidence of the distribution of radioactive material in various parts of the cell. No tumour-bearing rats were used for these studies since the tumours were neither considered suitable for fractionation nor did they appear to differ markedly from tissues such as the liver in respect of the amount of radioactivity taken up. It can be seen in Fig. 1 that in each experiment the specific radioactivities of the proteins are much higher in each cell fraction of the kidney than that of the liver.

TABLE II.—*Specific Radioactivities of Whole Liver, Liver Proteins and Blood after Administration of PAM*

Period of exposure	Experiment number			
	3 75 minutes (μc/g)	4 200 minutes (μc/g.)	5 24 hours (μc/g)	6 48 hours (μc/g)
Liver, whole tissue . , .	0·037	0·059	0·014	0·010
Liver, proteins (percentage of total radioactivity in proteins)	0·035 (13%)	0·040 (9%)	0·027 (27%)	0·023 (32%)
Blood	0·023	0·015	0·009	0·007

Dose : 10 mg./kg.
Specific or total radioactivities in μc/g. fresh tissue or protein fraction.
Values for whole tissue calculated from results of radioactive assays of freeze dried samples.

Reaction with proteins in vitro

    Since some of the " free " radioactive material present in tissues *in vivo* would most likely react after death of the rat during the time taken to separate and isolate cell fractions the amount of radioactivity bound to the proteins of tissue suspensions after exposure to PAM *in vitro* was determined. In Table III are

TABLE III.—*Percentage Radioactivity Recovered with Proteins after Addition of PAM to Liver Tissue Suspension of Plasma Albumin Solution*

Temperature	PAM added (μc)	Radioactivity (% of total) recovered in							Plasma albumin after	
		Liver proteins after								
		0 min.	30 min	80 min.	105 min.	6 hr.	20 hr.		80 min.	20 hr.
4° .	0·1 .	0·6	—	—	—	0·6*†	1·7		—	0·8
	— .	1·0	—	—	—	0·7*	—		—	—
	0·5 .	—	—	—	0·5	—	—		—	—
	1·0 .	—	—	—	—	—	1·3		—	1·0
25° .	0·1 .	0·4	0·5	—	—	—	—		—	—
37° .	0·1 .	—	—	2·4	—	—	8·7		2·5	11·0
	1·0 .	—	—	3·2	—	—	11·0		2·0	11·0
	— .	—	—	—	—	—	11·0		—	—

Liver tissue suspensions (1 in 5) or plasma albumin solution (2·5 per cent w/v) 5·0 ml for each experiment ; solutions of PAM (Na salt) 1·0 ml.
* Cold TCA and lipid extraction only.
† Total volume = 11·0 ml.

shown the effects of temperature, amount of PAM (Na salt) and of duration of reaction on the radioactivity recovered in the final protein precipitate. Similar experiments were also performed with bovine plasma albumin. It can be seen that at low temperatures, the duration of the reaction, i.e. the period between addition of PAM and of TCA, slightly affects the amount of radioactivity recovered only after 20 hours ; but a ten-fold difference in the amount of PAM added had no effect. However at 37°, the proportion of the radioactivity recovered goes up with length of time of reaction. These differences may be due either to an increased reactivity of PAM with the proteins at 37°, or to the presence of products of reaction which bind to proteins more readily than PAM itself.
    In an attempt to decide between these two possibilities samples of PAM hydrolysed in 0·155 M-NaHCO₃ replaced PAM in further otherwise identical

values. The use of lower concentrations of performic acid down to one two-hundredth of that normally taken resulted in the removal of between 15 and 50 per cent of the specific radioactivity. Formic acid alone had little effect; the decrease in the specific radioactivities was usually not above 10 per cent.
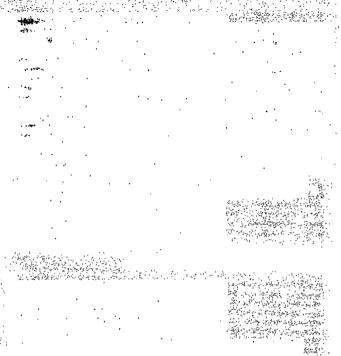
The tests using sodium hydroxide under three different conditions (Table IV) can be seen to produce a drop in the specific radioactivities to between 63–76 per cent of the control values. The effects of formic and performic acid and of sodium hydroxide were also examined on protein samples from experiments in which PAM or a hydrolysate had been added to tissue suspensions or a solution of plasma albumin. Most samples lost about 20 per cent of their specific radioactivity on treatment with formic acid. In experiments with PAM performic acid, reduced the specific radioactivity of liver samples to 29–49 per cent and of plasma albumin samples to 64–74 per cent of control values. Proteins from liver tissue suspension which had been allowed to react with a hydrolysate of PAM retained 54–68 per cent of the specific radioactivities of the control values. The protein samples from all experiments *in vitro* were found to have a similar resistance to the action of sodium hydroxide as those taken from injected animals.

Chromatography of protein hydrolysates

In addition to the examination of the effect of these reactions on the specific radioactivity of the proteins evidence was sought of the possible identity of the radioactive material by chromatography of hydrolysates of proteins. For this purpose samples from the kidney were used which were considered to have a specific radioactivity great enough for subsequent autoradiography. Much of the radioactive material stayed, however, at the origin of the chromatogram, the remainder moved as a streak for a short distance only. The happened with all solvent systems used except in one set of experiments when two spots could be detected in the streak after a run in methylethylketone-water-acetic acid. No radioactivity was ever detected in the phenylalanine and tyrosine spots. These observations were then compared with the chromatographic behaviour of PAM under various conditions of hydrolysis. Hydrochloric acid (6N), 0·155 M-sodium bicarbonate (pH 8·6), 0·145 M-sodium chloride (pH 5·8) and 0·1 M phosphate buffer (pH 7·1) yielded between six and twelve spots most of which were slow moving ($R_f < 0.3$) and linked by a streak to the origin. In addition a few medium fast spots could often be detected. The freshly prepared sodium salt of PAM moved fastest of all. The pattern of the spots was very similar whatever the method of hydrolysis used. The radioactive spots were almost always positive to ninhydrin and absorbed ultraviolet light.

DISCUSSION

The present investigation has shown that radioactivity derived from PAM is found in all the tissues examined and in all the cell fractions. It is often present to a greater extent in the protein fractions of the cytoplasm than of the cell nuclei. More radioactivity is bound to the protein fraction than to the nucleic acids. A large residual amount of radioactivity in the protein fraction withstands the attack of reagents which might be capable of breaking ester and ether bonds formed by interaction with the nitrogen mustard group. The possibility remains that PAM may have reacted with the proteins through peptide linkages either in

the microsomes had the highest specific radioactivities. In contrast, there was little difference in the specific radioactivities of the cell fractions of the liver.

3. The proteins always had a higher specific radioactivity than the nucleic acids separated from the same tissue.

4. PAM was found to react *in vitro* with proteins of liver tissue and with plasma albumin. Radioactivity was also bound to these proteins when in the presence of hydrolysate of PAM. It follows that the mode of binding of at least part of the radioactivity derived from PAM does not require the presence of the chloro-groups and also that it is not necessarily a metabolic process.

5. Chromatography of hydrolysates of protein samples showed no radio-activity in tyrosine or phenylalanine spots so that no conversion of PAM to one of these amino acids was observed.

REFERENCES

BERGEL, F., BURNOP, V. C. E. AND STOCK, J. A.—(1955) *J. chem Soc*, 1223.
Idem AND STOCK, J. A.—(1953) *Ann. Rep Brit Emp Cancer Campgn.*, 31, 6.—(1954) *J. chem. Soc.*, 2409.
BORSOOK, H, DEASY, C. L, HAAGEN-SMIT, A. J., KEIGHLEY, G. AND LOWRY, P. H.—(1952) *J. biol. Chem.*, 196, 669.
GOLOMBIC, C., FRUTON, J. S. AND BERGMANN, M —(1946) *J. org Chem.*, 11, 518.
JONES, A. S. AND MAR ⋅ C F (1051) *Biochim biophys Acta* 14 559.
KOLLER, P. C. AND VE   ⎫
KIRBY, K S —(1956a)   ⎬ 4, 359.
PETERSON, E. A. AND   ⎭
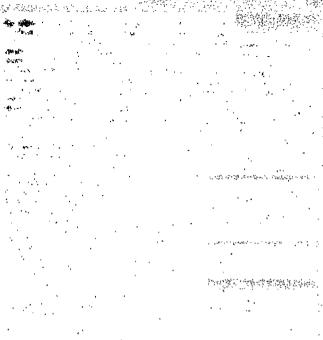
could be removed by the action of proteolytic enzymes, such as chymo-
trypsin, which has been found to retain its activity in the presence of
DNA (4). The effect of trypsin and chymotrypsin on the sedimentation
behavior of DNA has been examined.

EXPERIMENTAL

Sedimentation Experiments

The sedimentation experiments were performed using a Spinco analytical ultra-
centrifuge fitted with an ultraviolet light-absorption optical system The sedi-
mentation-coefficient distribution curves were calculated as previously described
(2). Seven different preparations of DNA from calf thymus were examined. In
each case the samples were dissolved in water at concentrations of 0.004–0 007%
DNA, and then sodium acetate was added to 0.1 M. Chymotrypsin and trypsin
were added to these solutions in amounts by weight approximately equal to the
amount of DNA The solutions were kept at 4°C. for 2–7 days and re-examined.
These were thought to be suitable conditions for the action of the enzyme, while
ensuring the stability of the DNA.

The addition of trypsin had no effect on any of the preparations in-
vestigated. The results obtained on the addition of chymotrypsin are
shown in Table I. With the three samples prepared by the detergent
method (5) (TNA 23, 32, and 33A) it resulted in definite changes in
the sedimentation characteristics. The distribution curves observed for
TNA 32 are shown in Fig. 1. It can be seen that in this case the addition
of chymotrypsin leads to an increase in the proportion of DNA with
sedimentation coefficients in the range $S = 16$–30 and a corresponding
decrease in the proportion with $S > 30$. Similar curves were observed

TABLE I

Preparation	Method of preparation	Average S in 0 1 M NaAc	Average S in 0 1 M NaAc + chymo-trypsin	Per cent protein	Per cent of DNA aggregated by protein
TNA 7	Chymotrypsin	20	20	—	0
15	incubation	21	21	0.5	0
TNA 23		27	25	—	25
32	Dodecyl sulfate	29	24.5	0 8	25
33A		20	20	0.2	8
33B*		21.5	21.5	0.08	0
J29	Amyl alcohol + chloroform	22.5	21	—	—
Cv5	—	19.5	19.5	—	0

* TNA 33A after two further treatments with detergent.

The Influence of Protein on Heterogeneity of DNA

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INTRODUCTION

In recent years many investigations have been made of the physical properties of deoxyribonucleic acid (DNA) solutions and very varied results have been obtained even with preparations from similar tissues (1). Only limited conclusions can be drawn from such results until methods of preparation are available which give rise to a product having reproducible properties. For this reason the investigation of the causes of variations in the physical characteristics of DNA is of importance.

It has also been found (2) in sedimentation experiments at low concentrations that DNA, as normally prepared from various tissues is extremely polydisperse, the range of sedimentation coefficients being from 10 S to 40 S or greater. That this heterogeneity is real has been shown by the fact that fractions having different sedimentation characteristics can be separated from one solution by sedimentation (3). The position and shape of the sedimentation-constant distribution curve is indeed quite variable with different samples of DNA, and in the first place it is necessary to find the origin of these differences.

One factor in this polydispersity is the possible aggregation of DNA particles. It has been shown (2, 4) that it is unlikely that aggregation is caused either by heavy-metal ions or by residual histone remaining in the preparation. Analysis of DNA preparations has shown that they contain small amounts (up to 1 %) of protein, which is not basic in character, and this may be responsible for aggregation by forming "cross-links" between two DNA particles. This protein is usually not entirely removed in many of the preparative procedures used, such as the precipitation of proteins by an anionic detergent. It was thought that it

treatment were unaffected by chymotrypsin. This shows that the aggregates which are broken down by chymotrypsin treatment are concentrated in the fraction having sedimentation constants >30.

The proportion of DNA which is cross-linked by protein can be estimated from the fraction of the material of which the sedimentation coefficients are displaced to lower values by the chymotrypsin treatment. This is shown shaded in Fig. 1. The estimates obtained are recorded in the last column of Table I.

The preparation CV 5, kindly given to us by Professor Sadron and Dr. Vendrely, was also examined. It has a very high molecular weight of 16.5×10^6 and, having a radius of gyration of only 2550 A,[1] is comparatively compact (7). It seemed possible that the compact form of the particles of CV 5 might be due to intramolecular cross-linking by residual protein, but it was found, however, that chymotrypsin had no effect on it.

Proteins Present in DNA Preparations

(a) *Analysis.* There is no simple analytical method of determining the residual protein in DNA preparations. In theory, it is possible to determine the amount from the ratio of N to P, but in practice it is impossible to determine this ratio with sufficient accuracy. We therefore adopted the laborious method of estimating separately all the amino acids formed after complete hydrolysis. This can be performed for all the amino acids present except glycine, which is produced in large quantities in the acid degradation of adenine and guanine, and was omitted from the summation.

The amounts of the amino acids were determined after hydrolysis for 24 hr. in a sealed tube at 110° with 6 N hydrochloric acid, by converting the amino acids formed into dinitrophenol derivatives by treatment with 1-fluoro-2,4-dinitrobenzene (FDNB) at pH 9 for 2 hr. at 40°C. The ether-soluble derivatives were estimated chromatographically by a combination of the methods of Blackburn and Lowther (7); and of Levy (8).

The results obtained (Table II) are summed to give the total amino acid present, excluding glycine, and indicate its character.

(b) *Nature of Protein.* To ascertain if any part of the residual "protein" was present as small peptides or even as amino acid nucleotides, a deoxyribonuclease digest of DNA was coupled with FDNB. A small

[1] Cf. TNA 7 with $M = 8.5 \times 10^6$ and radius of gyration 2440 A.
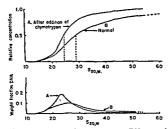
Fig. 1. The effect of chymotrypsin treatment on DNA sample J 32. Upper figure: integral distribution curves (weight fraction of DNA at any S is equal to the slope of the curve at this point). Dotted lines indicate weight-average sedimentation coefficient.

Lower figure: normal distribution curves showing weight fraction of DNA (between $S + 1$ and $S - 1$). Shaded area is equivalent to fraction of DNA-protein aggregates.

for TNA 23 and 33A although in the latter case the change in the distribution was much smaller. The amount of protein in TNA 33A was only 0.2% and this was reduced still further by two more treatments with the detergent. The sedimentation curve of the product (TNA 33B) was not affected by chymotrypsin. The two samples (TNA 7 and 15) had already been treated with chymotrypsin during the course of preparation (4). In these cases it was therefore expected that it would not have any further effect. This was borne out by the experiments. Similar experiments were also performed using a sample of DNA prepared by the Sevag method (6) (TNA 29). It has previously been shown that the Sevag method produces a DNA which sediments more slowly than that produced by the detergent method (2). The addition of chymotrypsin caused a definite change in the distribution curve. The behavior of the Sevag product is thus still influenced by the presence of protein.

The change in the distribution curves caused by chymotrypsin can be attributed to the severance of protein cross-links holding aggregates of DNA particles together. The correctness of the view has been confirmed by centrifuging in a swinging bucket rotor for a time which was calculated to be sufficient to remove the larger particles with $S > 30$. The sedimentation characteristics of the supernatant left after this
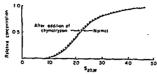
FIG. 2. The effect of chymotrypsin treatment on DNA sample J 33B. Integral distribution curves.

their asymmetry, such as might result from the breaking of intramolecular cross-links. In the latter case the viscosity of the solution would be increased. It was found, however, that the action of chymotrypsin reduces the viscosity of the solution, i.e., reduces the size of the particles. It would appear that the cross-links are mainly intermolecular and not intramolecular. It may be noted that the result of reducing the amount of aggregation by reaction with chymotrypsin is to reduce the differences observed between different preparations. The amount of protein remaining in DNA can be reduced, as in TNA 33B, to a very small value. As this preparation still shows a spread of sedimentation coefficients from $S = 10$ to $S = 40$ (Fig. 2), and is unaffected by chymotrypsin, it follows that there is a considerable amount of heterogeneity, even in preparations which have less than 0.1 % protein, which would appear to be due to heterogeneity of the DNA particles themselves. A serious difficulty that remains is that we have no method of determining whether any residual aggregation occurs through the small remaining amounts of peptides or proteins which are not susceptible to attack by chymotrypsin. The estimates of the proportion of DNA which is aggregated in this way must therefore, for the moment, be regarded as minimum values.

Table II shows that most of the amino acids commonly found in proteins occur in minute amounts in highly purified DNA preparations. The high proportion of aspartic and glutamic acids and the generally low proportion of lysine (as well as the inequality of the alanine and lysine values) indicate that this material, if protein, is not histone. The absence of hydroxyproline also indicates that it is not a contaminating connective tissue protein, such as collagen.

It must be borne in mind that since we are dealing here with a minute amount of protein remaining in a nucleic acid which was originally ob-

TABLE II

Amino Acids Found in Hydrolyzates of Various DNA Samples (Molar Ratios)

For comparative purposes the alanine value has in all cases been taken as 10.

Cystine, methionine, and hydroxyproline were not seen in any hydrolyzate, and tryptophan and amide were not determined.

Amino acid	TNA 15	TNA 32	TNA 33A	TNA 33B
Alanine	10	10	10	10
Aspartic acid ⎰	23	12	48[a]	18
Glutamic acid ⎱		11		
Glycine	—	—	—	
Valine	6	8	11	⎰18
Leucines	10	17	13	⎱
Phenylalanine	2	4	3	0
Tyrosine	0	0	+	0
Serine	7	9	10	7
Threonine	9	4	?	4.5
Proline	7	11	6	2
Lysine	4	5	2	0
Arginine	11	7	15	0
Histidine	9	6	?	0
Total as % of DNA	0 5	0 8	0.2	0.08
N/P ratio of DNA	1 64	1.69	1 61	—

[a] It is possible that the aspartic-glutamic acid spot contained the threonine in this preparation

insoluble yellow precipitate was formed which contains all the amino acid types found in a total hydrolyzate of the undigested DNA, and as it also contains virtually no phosphorus, it is probable that it is dinitrophenylated 'protein or proteins The supernatant material, containing all the nucleotides from the digest gave practically no amino acids on complete hydrolysis (glycine excepted). It is suggested therefore that the amino acids found in these DNA preparations are largely present as protein.

DISCUSSION

The results of the sedimentation experiments given in Table I clearly demonstrate the marked effect that small amounts of residual protein can have on the physical properties of DNA. The observed decrease of the sedimentation coefficient produced by chymotrypsin treatment may be due either to a decrease in size of the particles or to an increase in

Fractionation of microsomal proteins by a non-ionic detergent

A considerable amount of evidence[1,2,3] has been accumulated to show that the combined proteins of the microsome fraction incorporate labelled amino acids more readily than those of any other cell fraction It would be of great interest to know the nature of the proteins into which this incorporation occurs. Attempts have been made to obtain protein constituents of microsomes by extraction with sodium deoxycholate[4] or sodium chloride followed by sodium hydroxide[5] The protein fractions which remained associated with ribonucleic acid (RNA) after such treatments were usually found, after short periods of incorporation, to have higher specific radioactivities than those devoid of RNA.

aesthesia induced by pentobarbitone sodium In experiments designed to examine the incorpo-

were centrifuged from the mitochondrial supernatant liquid which had been diluted with 0.7 volumes of 0.25 M sucrose The microsome pellets were resuspended with the aid of a polythene pestle in Lubrol W dissolved in 0.25 M sucrose. They were then centrifuged at 105,000 × g for 2 h to give the "detergent pellet", which had the same appearance as the microsome pellet. The supernatant liquid was sometimes freeze-dried and excess detergent was extracted with acetone All samples were then analysed for RNA and total proteins according to LITTLEFIELD *et al.*[6]. Lipid phosphorus was determined on the chloroform–ethanol–ether extract The final pro-
tein residue was assayed for radioactivity on polythene planchets (1 cm²) in an end-window counter connected to a scaling unit (Type 100 C, Panax Equipment Ltd.).

Fig. 1 shows the effect of different concen-
trations of Lubrol W on the composition of the detergent pellet It can be seen that, at all con-
centrations used, virtually all the RNA is re-
covered in the detergent pellet The proportion of protein remaining in this pellet reached a plateau at a value of approximately 50% in the presence of detergent concentrations between
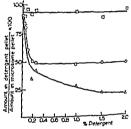
Fig. 1. Fractionation of microsomal con-
stituents by Lubrol W. □—□ RNA; O—O protein; △—△ lipid phosphorus.

pellet In the detergent pellet the amount of protein found was approximately 4½ times the amount of RNA It may be noted that this amount of protein is considerably greater than that which LITTLEFIELD *et al.*[4] observed after treatment of the microsome pellet with sodium deoxycholate

Table I shows the effect of the length of the period of incorporation on the specific radio-
activities of the proteins of the two microsome fractions in comparison with those of the micro-
somes as a whole and the final supernatant liquid. After periods of 6 min or longer the proteins of the fraction solubilized by Lubrol W had a specific radioactivity approximately twice that of those proteins which remained in association with virtually all the RNA of the microsome pellet It can also be seen that variations from 0.25% to 1.0% in the concentrations of detergent used did not affect the specific radioactivities of the protein of the detergent pellet or its supernatant

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tained in the form of nucleoprotein from a homogenate, it is possible that it represents contamination picked up, e.g., from the cytoplasm, during the preparation. It is clear, however, that the amount of amino acids or small peptides bound by covalent bonds present in DNA prepared by the dodecyl sulfate method of Kay, Simmons, and Dounce (5) from thymus gland, is extremely small (less than 0.1% in the case of TNA 33). This is in marked contrast to some results now appearing in the study of ribonucleic acid (9).

ACKNOWLEDGMENTS

SUMMARY

The effect of small quantities of residual protein on the sedimentation heterogeneity of DNA has been examined. It is found that treatment of the DNA with chymotrypsin reduces the fraction having high sedimentation coefficients, and it is concluded that the latter are due to protein–DNA aggregates. The amount and composition of the protein present prepared in various ways has been determined.

REFERENCES

1. SADRON, C., *Repts Congr intern. biochim. 3e Congr. Brussels, 1955*, p. 210.
2. SHOOTER, K. V., AND BUTLER, J. A. V., *Trans. Faraday Soc.* **52**, 734 (1956); SHOOTER, K V., AND BUTLER, J. A V., *Nature* **177**, 1033 (1956).
3. SHOOTER, K V , AND BUTLER, J. A. V., *In* "Chemical Basis of Heredity," (W. D. McElroy and B. Glass, eds.), p. 540. Johns Hopkins Press, Baltimore, 1955.
4. BUTLER, J A V , CONWAY, B. E , AND JAMES, D. W. F , *Trans. Faraday Soc.* **50**, 612 (1954)
5. KAY, E R. M , SIMMONS, N S., AND DOUNCE, A., *J. Am. Chem. Soc.* **74**, 1742 (1952)
6. GULLAND, J M , JORDAN, D O., AND THRELFALL, G. J., *J Chem Soc.* **1947**, 1129
7. BLACKBURN, S , AND LOWTHER, A. G , *Biochem. J.* **48**, 126 (1951).
8. LEVY, A L , *Nature* **174**, 126 (1954)
9. POTTER, J L., AND DOUNCE, A. L., *J Am. Chem. Soc.* **78**, 3078 (1956)

Physical Fractionation of DNA by Centrifugation and Other Processes

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A technique of using ultraviolet light absorption for the study of the sedimentation of nucleic acids at low concentrations (0.01–0.001%) has been described.[1,2] It was found that with all the samples studied the distribution of sedimentation coefficients extends over a considerable range ($S = 10$ to 40). This heterogeneity appeared to be a real characteristic of the materials examined, as shown by various tests that were applied. The samples investigated however differed among themselves, suggesting that the nature of the product is influenced by known and unknown factors in the method of preparation. A study has now been made of some of these variables and it is found possible to vary the distribution of sedimentation constants in the product by various procedures. If the distribution of sedimentation constants is real it should also be possible to effect a fractionation by sedimentation itself on a preparative scale. This has been achieved although unfortunately, owing to the very low concentrations which are necessarily employed, the amount of the fractionated material obtained is very small.

EXPERIMENTAL

(1) Preparative Fractionation of DNA in Swinging Bucket Rotor

A 0.007% solution of DNA in 0.2 M sodium chloride was centrifuged for 4.5 hours at 39,000 r.p.m. in the swinging bucket rotor developed by Spinco Corporation (using the Spinco analytical centrifuge). When deceleration was effected by the use of the brake it was found that stirring of the solution occurred; but, when the brake was not used so that deceleration occurred slowly under the influence of the frictional forces present, it was possible to avoid mixing.

The run was continued until the average concentration of DNA left in the solution was 0.001%, the greater part having sedimented to the bottom of the tubes, where it was present as a gel-like mass. The supernatant solutions were poured off from this gel, and after standing for 24 hours their sedimentation coefficient distribution curves were examined in the analyti-

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TABLE I

SPECIFIC RADIOACTIVITIES OF PROTEINS OF MICROSOMAL FRACTIONS AND SUPERNATANT LIQUID

| Body weight (g) | Period (min) | Concn. of detergent (%) | Microsomes | Specific radioactivities (μc/g) of proteins — Detergent treatment | | Pellet resuspended in Na deoxycholate | | Final supernatant liquid |
				Pellet	Supernatant liquid	Sediment	Supernatant liquid	
350	2*	0.25	0.71	0.90	0.46	1.95	0.39	0 11
335	6	0 25	0.94	0.78	1 33			0.25
		1.0		0 70	1 16			
230	10½**	0.25	2.22	1.34	2.91	1.43	1 38	0.68
333	10½	1.0	2.06	1.07	2 56			0.43
221	31½	0.25	4.59	2.47	5.40			1.53
		1.0		2.45	5 12			
		1 0		2.40	5 85			

Each rat received 0 2 mc/kg of DL-phenylalanine(3-14C) (2 mc/mmole) intraperitoneally, except.
 * Injection into tail vein under light ether anaesthesia.
** Injection into femoral vein under light ether anaesthesia.

negligible It can be seen that these ribonucleoproteins, not solubilized by sodium deoxycholate, were responsible for much of the high initial specific radioactivity of the detergent pellet In contrast, the lipoproteins of the detergent pellet had a lower specific radioactivity than the proteins of the supernatant liquid obtained by the treatment of the microsomes with Lubrol W. When the time of incorporation was extended to 10 5 min, the two protein fractions produced by the treatment of the detergent pellet with sodium deoxycholate had the same specific radioactivities.

An effect of the non-ionic detergent Lubrol W on microsomes is thus to produce a fraction whose proteins become readily labelled within a few minutes of the administration of phenylalanine-14C.

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[1] H. BORSOOK, C. L. DEASY, A. J HAAGEN-SMIT, G. KEIGHLEY AND P. H. LOWY, J. Biol. Chem., 187 (1950) 839.
[2] T. HULTIN, Exptl. Cell Research, 1 (1950) 376.
[3] E. B. KELLER, P. C. ZAMECNIK AND R B LOFTFIELD, J. Histochem Cytochem , 2 (1954) 378
[4] J. W. LITTLEFIELD, E. B. KELLER, J. GROSS AND P. C. ZAMECNIK, J. Biol. Chem , 217 (1955) 111.
[5] J. L. SIMKIN AND T S. WORK, Biochem. J., 65 (1957) 307
[6] F. W. PUTNAM, in H NEURATH AND K. BAILEY, The Proteins, Vol. II, Academic Press, New York, 1953. p. 824.
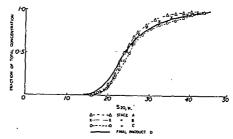
Fig. 2. Sedimentation coefficient distribution curves obtained at different stages of the preparation of DNA by the detergent method.
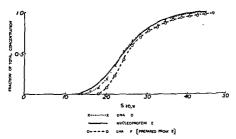
Fig. 3. Comparison of the sedimentation coefficient distribution curves for preparations of DNA and a nucleoprotein.

In another experiment (Fig. 3) the sedimentation curve obtained simply by dissolving a nucleoprotein preparation in 2.5 M sodium chloride is shown (curve E). In such a solution dissociation of the nucleoprotein into DNA and histone is almost complete[5] and the contribution of the protein to the absorption of the solutions is slight.

The distribution curve of this material is very similar to that of the DNA (e.g., the preparation D) of the previous experiment. DNA prepared from E by the detergent method was, however, deficient in some of the more slowly sedimenting fractions. This is probably due to loss of this material in the stage of precipitation with alcohol. It has in fact been found that DNA with low sedimentation constants is precipitated by alcohol comparatively slowly and in a powdery and not a fibrous form.
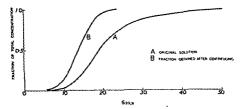
Fig. 1. Sedimentation coefficient distribution curves showing fractiona-
tion achieved after prolonged centrifuging.

cal machine (Fig. 1). It can be seen that, although there is still a range of
sedimentation coefficients in the supernatant, the average value of S is
only 14, as compared with 19 for the original solution. It is calculated that
under the sedimentation conditions mentioned no material with $S > 20$
should be left in the solution; the maximum value of S in the solution was
22, and only 4% of the DNA had values of $S > 20$. It may be concluded
that a sedimentation fractionation has been achieved which is in reasonable
agreement with that to be expected from the sedimentation coefficients.

(2) Influence of Method of Preparation

(a) Detergent Method of Kay, Simmons, and Dounce[3]

In these experiments the nucleoprotein of calf thymus tissue was pre-
pared as described by Shooter, Davison, and Butler,[4] and left at the stage
of a suspension in 0.14 M sodium chloride. The nature of the product (dis-
solved in 2.5 M sodium chloride) was examined in the ultracentrifuge at the
following stages:

(1) To the suspension 5% detergent (sodium dodecyl sulfate) in 45%
alcohol was added, followed by precipitation of the protein detergent com-
plex with sodium chloride at 1 M and centrifuging. Solution A is the
supernatant.

(2) The DNA in this solution was precipitated with alcohol and washed
and dissolved in water. Detergent was again added followed by NaCl to
1 M as before. The supernatant after centrifuging was used for solution B.

(3) The DNA was precipitated with alcohol and washed and redissolved
in water and finally salt was added to 0.14 M (solution C).

(4) This solution was centrifuged and the DNA was precipitated with
alcohol and washed and dried in a vacuum desiccator. This product was
used for solution D.

It can be seen in Figure 2 that these four products differ very little in
their sedimentation distributions, so that the character of the product is
hardly affected at all by the purification procedures used.
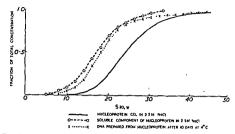
Fig. 5. Comparison of the sedimentation coefficient distribution curves for the dispersed and gel phases of nucleoprotein.

was not a simple process of dispersion of the gel, but involved some enzyme action. Observations made on solutions obtained both from the gelatinous material (isolated by centrifuging) and on the dispersed material in 2.5 M NaCl (0.006% DNA) are shown in Figure 4. It can be seen that the sedimentation coefficients of the DNA in the gel phase are much higher than those of the dispersed substance. However, it can be seen from Figure 3 that DNA made from this gel by the detergent method has a similar sedimentation coefficient distribution. On standing until "liquefied" the gel material in 2.5 M NaCl gives a similar pattern to that of the original dispersed material. It is therefore reasonable to conclude that in the dispersion a process of breakdown or disaggregation of the DNA itself is occurring.

DISCUSSION

It is evident that physical fractionations of DNA can be performed both by centrifugation and in the course of various preparative procedures. This may help to explain the very wide variation of physical properties observed by different workers with apparently similar preparations. The detergent method of Kay, Simmons, and Dounce[3] gives a product with characteristics similar to those of the DNA in the nucleoprotein. On the

TABLE I

DNA preparation	Guanine, %	Adenine, %	Cytosine, %	Thymine, %
Prepared by Sevag method..	24 6 ± 0 3[a]	29 2 ± 0 3[a]	21 5 ± 0 4[a]	24 7 ± 0 6[a]
From Sevag residues.....	23.9	28 5	21.2	26 3
From nucleoprotein gel[b]	24.6	28 7	22.3	23 3
From dispersed nucleoprotein.	23 7	29.7	21.2	25.4
Unfractionated......... .	24.7	29.0	20 8	24 8
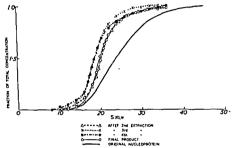
[a] Standard deviation.
[b] This preparation contained 1.2% uracil, i e., 4.9% ribonucleic acid

(b) Sevag Process[6]

In this process the nucleoprotein was suspended in 0.14 M sodium chloride, and the concentration of salt increased to 2 M. The solution was then homogenized with a equal volume of a 10:35 mixture of amyl alcohol and chloroform for 30 sec. in a Waring Blendor and centrifuged. The clear supernatant was collected and the lower layers containing denatured protein were rejected. The alcohol–chloroform treatment was then repeated several times.

The products after the second, third, and fourth extractions were examined, as before, in the ultracentrifuge in 2.5 M sodium chloride.

It can be seen (Fig. 4) that a definite fractionation occurs in that the

Fig. 4. Sedimentation coefficient distribution curves obtained at different stages of the preparation of DNA by the Sevag method.

material of high sedimentation constant is lost in the first two Sevag treatments, but later, similar treatments caused no significant change. The latter result must mean that blending with alcohol–chloroform does not by itself produce progressive changes in the DNA. It is probable that the fractions of DNA with high values of S are selectively removed with the denatured proteins and are not extracted by the salt solution. To test this a preparation of DNA was made by the detergent method from the precipitate obtained in the first treatment. It was found that its average sedimentation constant was 2.5 units greater than the final Sevag product.

(c) Separation of Gel and Disperse Phases of Nucleoprotein

It was found[4] that when calf thymus nucleoprotein is dialyzed against water to remove salt it forms a gelatinous material which contains both persistent gel "islands" and more dispersed particles. If the gel is allowed to stand at 4° it eventually becomes dispersed, but it was thought that this

References

1. Shooter and Butler, *Nature*, 175, 500 (1955).
2. Shooter and Butler, *Trans. Faraday Soc.*, 52, 734 (1956).
3. Kay, Simmons, and Dounce, *J. Am. Chem. Soc.*, 74, 1724 (1952).
4. Shooter, Davison, and Butler, *Biochim. et Biophys. Acta*, 13, 192 (1954).
5. Butler, Davison, and James, *Proc. Biochem. Soc*, 54, xxi (1953).
6. Gulland, Jordan, and Threlfall, *J. Chem. Soc.*, 1947, 1129.
7. Wyatt, *Biochem. J.*, 48, 584 (1951).
8. Chargaff, Crampton, and Lipshitz, *Nature*, 172, 289 (1953)
9. Chargaff, *Trans. Faraday Soc.*, 50, 293 (1954).
10. Lucy and Butler, *Nature*, 174, 32 (1954).

other hand the Sevag process[6] when used in the way described produces a marked fractionation. Table I shows, however, that this physical fractionation is not accompanied by any significant changes of the base composition of the products. The analysis of the DNA was performed using the method of Wyatt.[7] This is of interest as Chargaff[8,9] found that by extracting the Sevag precipitate with salt solutions of increasing strength it was possible to effect a chemical fractionation of DNA, the earliest fractions extracted being relatively rich in guanine and cytosine. Butler and Lucy[10] also found that such a fractionation can also be performed by successive extractions with a constant concentration of sodium chloride (0.6 N). This was not accompanied by any great degree of physical fractionation. It appears that the extraction with sodium chloride at a concentration as high as 2 M carried out here brings about the solution of a wide range of chemical fractions, but there is still a fraction with high sedimentation constants which cannot be removed from the Sevag precipitate.

The experiments carried out on the nucleoprotein gels indicate that the dispersion of the gel islands is not a simple process of dissolution but involves some measure of degradation of the DNA. This may well be an enzyme process, probably brought about by tissue deoxyribonucleases. Analysis of the DNA prepared from the nucleoprotein gel and from the dispersed material originally present (see Table I) shows that they do not differ significantly in base composition.

SUMMARY

(1) By sedimentation at low concentration, DNA fractions can be obtained which have appreciably different average sedimentation coefficients.

(2) The sodium dodecyl sulfate method of preparation gives a DNA with a similar distribution of sedimentation coefficients to that of the original nucleoprotein. With the Sevag procedure on the other hand a marked fractionation occurs, the material extracted by salt having appreciably lower sedimentation coefficients than the material that is not extracted.

(3) Nucleoprotein gels have been shown to contain both dispersed particles and a gel phase. The DNA of the dispersed material has a lower range of sedimentation coefficients than that present in the gel phase. The dispersion of the gel phase that occurs on standing is accompanied by a degradation of the DNA.

The deoxyribonucleic acid of the membrane fraction may be associated with some of the small spherical granules that can be seen attached to the surfaces of the cytoplasmic membranes after disruption by osmotic shock. We have found that treatment at 30° for 60 min. with 1 per cent aqueous sodium ethylenediamine tetra-acetate (pH 7) digests these bodies, at the same time solubilizing the deoxyribonucleic acid and some of the protein. Tracer studies were then performed as before with whole cells, but with the addition of this further fractionation procedure. The proteins from the resulting three fractions (membrane, cytoplasm, and 'deoxyribonucleic acid-protein') were then assayed, the results being shown in Fig. 2.

These results therefore show that the initial observable incorporation of [1-¹⁴C]-glycine into proteins occurs in this case in the cytoplasmic membrane. On the other hand, the protein associated with the deoxyribonucleic acid has the lowest specific radioactivity of the three fractions at all but the shortest periods of incubation, and even then is below the level found in the membrane fractions.

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[1] Weibull, C., J. Bact., 66, 688 (1953).
[2] McQuillen, K., Biochim. Biophys. Acta, 17, 382 (1955).

Sites of the Incorporation of an Amino-acid into Proteins of *Bacillus megaterium*

WHEN the digestion of *B. megaterium* is carried out with lysozyme in suitable media of high osmotic pressure, the rod-shaped cells are converted into from one to four spherical protoplasts[1]. It has been shown by McQuillen that these protoplasts are capable of growing and dividing, and of effecting the synthesis of protein, nucleic acids, and adaptive enzymes[1]. If the medium in which the protoplasts are suspended is diluted, the cytoplasmic membranes surrounding the protoplasts burst and much of the cytoplasmic constituents is liberated into the medium[1]. This liberated cytoplasm contains no particles equivalent to the mammalian microsomes, ultracentrifugal analysis having shown that not more than 10 per cent of the material has a maximum molecular weight of

Fig 1 Incorporation of [1-¹⁴C]-glycine into the protein of cytoplasmic and membrane fractions derived from protoplasts of *B. megaterium*

200,000, the residue being of much lower molecular weight [...]
cel [...]
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[...]
system for the study of protein synthesis in short time-intervals as it is possible to stop the process by pouring on to ice, thereby simultaneously cooling and bursting the cells with the consequent disruption of the cellular structure. Protoplasts were prepared from whole cells by aerobic incubation at 30° with lysozyme (0·2 mgm./ml.) in a glucose/ammonia/salts medium containing 0·5 *M* sodium citrate and 1 per

cent casein digest. When the conversion was complete, the protoplasts were harvested and resuspended in an inorganic salts medium containing 1 per cent glucose and 0·5 *M* potassium dihydrogen phosphate, After aerobic incubation for a further 1 hr. [1-¹⁴C]-glycine was introduced, the incubation continued, and samples removed and poured on to ice after various intervals. The membrane fraction from the burst cells was sedimented and the protein precipitated with trichloroacetic acid from the sediments and from the supernatant cytoplasmic fraction. Lipids and nucleic acids were removed by the usual washing procedures, and the radioactivity of the acetone-dried protein fraction was measured. The results (see Fig. 1) show that in short periods of incubation the membrane fraction becomes radioactive to a greater extent, though afterwards the cytoplasmic fraction becomes more highly labelled.

The difficulty of preparing large quantities of protoplasts capable always of incorporating the amino-acids into protein at measurable rates caused us to consider a modification of the above procedure. It was then found that similar results could be obtained by reversing the order of the incorporation and lysozyme digestion stages. In this case whole cells were incubated aerobically in the usual growth medium (glucose/ammonia/salts, pH 7), to which the radioactive amino-acid was added and samples taken as before. After sedimentation, the chilled whole cells were resuspended in water and lysozyme added to digest the cell wall at 0°. In this way lysozyme and the less radioactive cell wall 'proteins' did reduce somewhat the radioactivity observed in the cytoplasmic fraction, but the results were qualitatively similar to those observed with protoplasts.
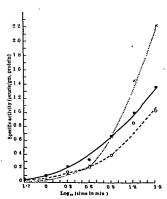

Fig 2. Incorporation of [1-¹⁴C] glycine into the proteins of cellular fractions derived from whole cells of *B. megaterium*. The cells (5 mgm./ml.) were incubated aerobically in an inorganic salts medium (pH 7·0) containing 1 per cent glucose, and sampling was carried out as described in the text after the addition of [1-¹⁴C] glycine (0·05 μc/ml.). ●, Membrane; ×, cytoplasm; ○, deoxyribonucleic acid protein

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THE PHYSICAL CHEMISTRY OF DEOXYRIBOSENUCLEIC ACID

K. V. Shooter

I. INTRODUCTION

SEVERAL reviews (JORDAN, 1951; CHARGAFF and DAVIDSON, 1955; DAVISON *et al.*, 1954; ZAMENHOF, 1956; ALLFREY *et al.*, 1955; BUTLER and DAVISON, in press) dealing with the chemistry of deoxyribonucleic acid (DNA) have appeared in the last few years. The recent elucidation of the structure of the molecule has however opened up new fields for investigation. In this article, in addition to reviewing the recent work on the physical chemistry of DNA, an attempt has been made to co-ordinate some of the earlier work in terms of the molecular structure.

When dealing with highly polymeric materials of biological origin, it is of great importance to be able to determine the extent of the damage or degradation which is produced by the methods of preparation. In the case of DNA the work on the transforming principle has provided an extremely valuable yardstick in this respect (cf. the review by ZAMENHOF, 1956). It has been shown that the two most commonly used methods of isolating DNA from the nucleoprotein complex, treatment with amyl alcohol and chloroform (SEVAG *et al.*, 1938; GULLAND *et al.*, 1947a; McCARTY and AVERY, 1946) and treatment with detergent (KAY *et al.*, 1952; ZAMENHOF *et al.*, 1953), do not destroy the biological activity of transforming DNA. It seems probable therefore that DNA prepared from other sources using these methods can be assumed to be biologically intact. Further work has shown that the biological activity of transforming DNA can be destroyed in many ways, e.g. by short exposure to the action of deoxyribonuclease (AVERY *et al.*, 1944), exposure to ultraviolet light (ZAMENHOF *et al.*, 1954b), drying (AVERY *et al.*, 1944), removal of salt from the solution (AVERY *et al.*, 1944), by change of pH, or by heating (ZAMENHOF *et al.*, 1953; AVERY *et al.*, 1944). In the majority of cases it has also been found that the loss of activity is associated with measurable, irreversible changes in the physicochemical properties of the DNA. From experiments of this nature the criteria by which the extent of degradation and denaturation of a given sample of DNA can be characterized are being evolved. The results of these experiments and the correlation of the observed physicochemical changes with changes in the structure of the molecules are discussed later in this review.

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Molecular Weights and Physical Properties of Deoxyribonucleic Acid

IN the measurements of the molecular weight from light scattering and of other physical properties of deoxyribonucleic acid, an extraordinary variety of results has been obtained[1], even with samples prepared from a single source, namely, calf thymus. To some extent this might be accounted for as a result of fractionation of the material resulting from its physical heterogeneity, which has been demonstrated by Shooter and Butler[2], and by Schachman et al.[3]. It may also arise from small amounts of protein impurity, which have been shown[4] to affect the physical properties to a marked extent. In order to

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solutions of calf thymus deoxyribonucleic acid treated in various ways. It has been found that centrifuging as a means of clarifying the solutions is made much more effective . . . . to 0·04 per ce. . . . ing for 18 hr. . .
The difference must be due to the greater viscosity of the stronger solution. The light-scattering molecular weight, especially of the more turbid samples, is markedly reduced by centrifuging in this way at the lower concentration, without any great change of either the intrinsic viscosity or of the sedimentation coefficients. This must mean that the component which gives rise to high turbidities in the unclarified . . . . vis-
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. . . . solution (J40) with molecular weight 4 × 10⁶ reduced the light scattering to 20 per cent of its original value; while on a turbid solution (J38), having an apparent molecular weight of 17 × 10⁶, the light scattering value was reduced by only 40 per cent, that is, to 10 × 10⁶.
The results obtained with a number of preparations when treated in this way now agree very well with each other in most cases (Table 2), and in the exception (J38) it has been shown that the high value

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a factor of 3 than that determined from the intrinsic viscosity and mean sedimentation coefficient by the

Table 1. EFFECT OF CENTRIFUGING DEOXYRIBONUCLEIC ACID SOLUTIONS FOR 18 HR. AT 20,000g AT DIFFERENT CONCENTRATIONS

Sample	Concentration (per cent)	Yield of deoxyribonucleic acid in supernatant	$M_L \times 10^4$	$[\eta]$	S_s
J 31 C	0·1	88	>20	35	27
	0·04	66	3·0	35	27
J 34	0·1	92	12·2	34	25
	0·04	61	3·3	34	25

Table 2. PHYSICAL CONSTANTS OF DEOXYRIBONUCLEIC ACID SOLUTIONS AFTER CENTRIFUGING FOR 18 HR. AT 20,000g AT 0·04 PER CENT

Specimen of deoxyribonucleic acid	$M_L \times 10^4$	$[\eta]$	S_s	$M_{s,\eta} \times 10^4$
31C	3·0	35	27	10
33	3·3	33	25	8·5
36	3·3	33·4	28	10·8
40	4·0	50	22	9·0
34	3·3	34	25	9·0
38	17	47	23	9·5

equation of Mandelkern et al.[5]. The cause of this discrepancy may be (1) failure of the light scattering theory when applied to very long rods ; (2) the effect of the heterogeneity on the calculation from the intrinsic viscosity and sedimentation. These possibilities require further study.

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[1] Reichmann, M. E., Rice, S. A., Thomas, C. A., and Doty, P. J. . . . .

provides an explanation for the results obtained by CHARGAFF *et al.*
Further support for this type of structure was provided by the titration
studies of GULLAND, JORDAN, and TAYLOR (1947) and of COSGROVE and
JORDAN (1949) who had shown that there was a marked hysteresis
between the forward titration curve from pH 7 and the back titration
curve from acid or alkaline pH. This difference in the titration curves
was attributed to a non-random form of hydrogen bonding between
the titratable groups of the bases. In terms of the WATSON and CRICK
model it *is* now proposed (JORDAN, 1951) that the hydrogen bonds
between the bases begin to break when the pH is raised above 11 or
lowered below 5 resulting in an irreversible breakdown of the structure
and a liberation of the groups which normally would titrate in the pH
range 5–11. Once the structure has been destroyed, hydrogen bonding
occurs at random and the titration curves become reversible.

Detailed analysis of the diffraction pictures (FRANKLIN and GOSLING,
1953a,b; WILKINS *et al.*, 1953b; FRANKLIN and GOSLING, 1953,
1955; FEUGHELMAN *et al.*, 1955) has corroborated the essential features
of the model proposed by WATSON and CRICK though some modification
of the dimensions of the molecule was found to be necessary. (For
general reviews see JORDAN (1951) and CARLISLE and BERNAL, 1955.)
The dimensions and characteristics of the two main forms can be
summarized as follows. In the crystalline A form the two polynucleo-
tide chains form helices 18 Å in diameter spaced 14 Å apart along the
helix axis. The pitch of the helix is 28·1 Å and there are eleven nucleo-
tides on each chain per complete turn. The pairs of bases form a rod
inclined at an angle of 65° to the axis of the helix. In the B form of the
molecule the pitch of the helix increases to 33·1 Å and there are ten
nucleotides on each chain per complete turn. The pairs of bases form
rods passing through and lying perpendicular to the axis of the helix.

The analysis of the density of DNA in the A form together with the
estimates of the size of the unit cell have confirmed that the
structure contains two polynucleotide chains. Corresponding calcula-
tions for the B form suggest a three- rather than a two-chain structure.
It seems unlikely however that a reversible change between a three-
and a two-chain structure would occur so that it must be concluded
that the B form contains some units possessing a more compact
structure than the rest. Calculations of the Fourier transform have
corroborated that the B form is a twin chain molecule (FEUGHELMAN
et al., 1955). It must be emphasized that the measurement of the
intensity of the background scattering has shown that only 50–60 per
cent of the DNA exists in a crystalline structure. It may also be noted
that recent estimates of the ratios of the two pairs of bases are not
exactly equal to unity (LUCY and BUTLER, 1954) though this may be
in part due to experimental errors.

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II. THE STRUCTURE OF THE MOLECULE

X-ray diffraction experiments using filaments drawn from concentrated solutions have shown that DNA can exist in three main forms (FRANKLIN and GOSLING, 1953a,b). At 75 per cent relative humidity it possesses a highly crystalline, stable structure (A). At higher humidities it is paracrystalline (structure B). At lower humidities the structure becomes disordered. The changes in structure involved are reversible, the interconversion of the three forms being achieved by varying the humidity. Since in the B form each molecule is surrounded by a sheath of water molecules and is hence relatively free from the interactions of its neighbours, it is this form which approximates most closely to the structure of the molecule in solution.

In 1952, FURBERG (1952) proposed that DNA existed in the form of single strand helixes with the bases stacked on top of and parallel to each other, 3·4 Å apart. ASTBURY (1947) had suggested earlier that the observed 3·4 Å spacing represented the distance between the bases. This single strand structure does not however account very well for the stability of the helix at high humidities. PAULING and COREY (1953) proposed a three strand helical model in which the phosphate groups formed the central core. The work of FRANKLIN and GOSLING (1953a) on the A and B forms of DNA has however shown that the phosphate groups are accessible to water and must therefore be on the outside of any proposed structure. WILKINS et al. (1953a,b) and FRANKLIN and GOSLING (1953a,b) have analysed the diffraction patterns and have confirmed that the molecules exist in a helical configuration. It was also shown that the same diffraction pattern was obtained with preparations of DNA from a very wide variety of sources (WILKINS et al., 1953a,b). Similar patterns were observed for nucleoprotamine, nucleoprotein, dried sperm, and centrifuged T2 bacteriophage (WILKINS et al., 1953a), indicating that no major change in structure is associated with the removal of protein during the preparation of the DNA. Further evidence of a degree of uniformity between samples of DNA from many different sources was obtained by CHARGAFF and his colleagues (1955, 1953) who found that although the relative proportions of the purines to the pyrimidines differed widely the ratio of the amounts of adenine to thymine and of guanine to cytosine were close to unity. On the basis of this evidence WATSON and CRICK (1953, 1954) proposed a structure for the DNA molecule involving two polynucleotide chains with the phosphate groups on the outside and the bases pointing inwards. Analysis of the possible ways of pairing the bases showed that hydrogen bonding between adenine and thymine and between guanine and cytosine would give a regular arrangement with a constant diameter of the helix of 20 Å. The structure thus

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more is known about the shape of the particle and the degree of heterogeneity of the preparations these results must be considered as tentative but the molecular weights calculated for calf thymus DNA $6-12 \times 10^6$, are of the same order of magnitude as those determined using other methods.

Most of the recent estimates of the molecular weight have been obtained from light scattering experiments. The results and the
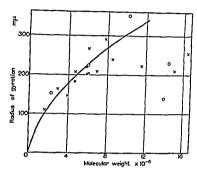

Fig. 1. Comparison of the molecular weight and radius of gyration of different samples of DNA.

× — Data taken from SADRON (1955).

O — Data taken from BROWN et al. (1955).

techniques and theory involved in these experiments have been described by STACEY (1956). SADRON (1955) has reviewed some of the results obtained and has listed the molecular weights of fifteen samples of calf thymus DNA ranging from $1 \cdot 9 - 16 \cdot 5 \times 10^6$. DOTY (1955) has reported that of fifty preparations of calf thymus DNA investigated in his laboratory, apart from some early specimens, e.g. DNA prepared by the method of SEVAG and GULLAND et al., for which $M = 3 \cdot 5 \times 10^6$, the majority of the results fall in the narrow range $M = 5 \cdot 8 - 7 \cdot 7 \times 10^6$. Preparations of DNA from other sources have been examined in the latter laboratory and have been found to have molecular weights close to 6×10^6 (e.g. DNA from pneumococcus $M = 7 \cdot 7 \times 10^6$; DNA from chicken erythrocytes $M = 4 \cdot 2 \times 10^6$). ROWEN and NORMAN (1954) have also reported that for DNA from $E.\ coli\ M = 7 \times 10^6$. From electron and deuteron bombardment studies FLUKE et al. (1952)

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III. Size and Shape of DNA Molecules in Solution

Early estimates of the molecular weight of DNA preparations were based on the combination of diffusion and sedimentation data obtained in the concentration range 1·0–0·02 per cent (JORDAN, 1951). These results are to some extent suspect since the sedimentation coefficient increases rapidly as the concentration is lowered and extrapolation to infinite dilution is difficult. The recent introduction of a centrifuge cell that holds a 3 cm column of solution has made it possible to measure sedimentation rates using the Philpot-Svensson optical system, at concentrations down to 0·005 per cent. It may be noted that at this low concentration the sedimenting boundary shows an appreciable degree of spreading (OTH, 1955). Further improvement has been obtained by the use of an ultraviolet light absorption optical system with which measurements can be made down to 0·001 per cent (SHOOTER et al., 1955, 1956a, SCHUMAKER et al., 1957). For calf thymus DNA, measurements at these low concentrations can be used to obtain fairly accurate weight average sedimentation constants at infinite dilution, and values in the range 21–29S have been reported (SHOOTER et al., 1956a). It has however been found that for some samples of DNA, e.g. from the bacteriophage D_4 of Salmonella enteritides, the dependence of sedimentation rate on concentration is so marked that even with measurements made at these low concentrations an accurate extrapolation to infinite dilution is not possible. Measurements at even lower concentrations, 10^{-5}–10^{-7} g ml^{-1} have been made by GOODGALL, RUPERT, and HERRIOTT (1957) for the transforming principle of Haemophilus influenzae using the partition cell method. The relative amounts of DNA in the two parts of the cell were determined from transforming activity and ^{32}P assays. They found that in this range of concentrations $S = 27 \pm 2·5$.

So far no extensive work on the measurement of diffusion coefficients at comparable concentrations has been done. JAMES (1954) has described an apparatus which can be used at concentrations at least down to 0·001 per cent and has given a preliminary value of D_0 of about 2×10^{-8} cm^2 sec^{-1} for calf thymus DNA in water and in 0·2 M NaCl. Combining this value with $S_0 = 20 \times 10^{-13}$ gives $M = 5 \times 10^6$. GOODGALL et al. (1957) have reported a preliminary value of D_{20} for the transforming principle of Haemophilus influenzae, measured by the sintered disc method, of 3×10^{-8} cm^2 sec^{-1}. Combining this with the sedimentation coefficient given above gives $M = 15 \times 10^6$. In the absence of suitable diffusion data molecular weights have been estimated from sedimentation and viscosity data using the equation of MANDELKERN et al. (1952) assuming the DNA particle to be a statistical coil (PEACOCKE and SCHACHMAN, 1954; SHOOTER et al., 1956). Until

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parameter termed the persistence length. The extension of this theory to deal with a heterogeneous system has not yet been achieved. It is clear that in order to resolve these ambiguities in the interpretation of the light scattering data independent measurements of the degree of heterogeneity of DNA are required.

IV. THE HETEROGENEITY OF DNA

Determinations of the degree of heterogeneity have been made from studies of the sizes of the individual molecules in electron microscope photographs and from investigations of the sedimentation characteristics of DNA solutions at low concentrations. Photographs taken by BAYLEY (1951) showed the DNA molecules as a polydisperse system of spheres with an average molecular weight of about 10^6. More recently photographs have been obtained showing the molecules as filaments (SCOTT, 1948; SCHUSTER et al., 1956) and from length measurements it has been estimated by WILLIAMS that $M_w/M_n = 1.6$. A typical electron microscope photograph of DNA is shown in Fig. 2. It may be noted that the filaments are slightly kinked or bent suggesting that the particle has some degree of flexibility.

Sedimentation experiments in the concentration range 0.01–0.001 have shown that the boundary exhibits a marked degree of spreading (SHOOTER et al., 1956a). Calculations of the curve showing the distribution of sedimentation coefficients indicated that the distribution did not change with time as sedimentation progressed and therefore that spreading due to diffusion or convection was negligible. Further demonstration of the stability of sedimenting boundaries at these low concentrations was obtained from a study of solutions of turnip yellow mosaic virus. Five measurements at concentrations between 0.3 per cent and 0.002 per cent gave the sedimentation coefficient as $114 \pm 3S$. The error is only very slightly greater ($\frac{1}{2}$ per cent) than the random error observed, when using the Spinco ultracentrifuge for repeated measurements at the same concentration. Since the virus molecule is approximately spherical in shape these results demonstrate that the stability of the sedimenting DNA boundaries at low concentrations is not associated with the fibrillar shape of the DNA molecule.

Curves showing the distribution of sedimentation constants at infinite dilution have been obtained by extrapolation using the method of OTH and DESREUX (1954). Fig. 3 shows the results obtained for five different preparations of DNA from calf thymus. The curves in the figure are the integral distribution curves, the weight fraction of DNA with any specific sedimentation constant being equal to the slope of the curve at that point. It is clear from these curves that DNA exhibits a marked sedimentation heterogeneity and also that significant

have calculated that for the DNA of pneumococcus transforming principle $M = 6 \times 10^6$. It has been suggested that DNA from all sources may have a molecular weight close to 6×10^6 but this suggestion seems a little premature in view of the much higher values reported by SADRON (1955) and SHOOTER et al. (1956) for calf thymus DNA and by GOODGALL et al. (1957) for transforming principle of Haemophilus influenzae. Other high values have been reported by BROWN, M'EWEN, and PRATT (1955) for DNA from E. coli ($M = 9.1 \times 10^6$) and from avian tubercle bacillus ($M = 13.5–14.4 \times 10^6$). A preliminary calculation of the molecular weight from sedimentation and viscosity data for DNA from bacteriophage D_4 of Salmonella enteritides has given $M = 26 \times 10^6$ (BARBU et al., in press).

A comparison of the properties of different preparations of DNA can be obtained from a plot of the molecular weight against the radius of gyration of the molecule (SADRON, 1955). Both of these quantities can be obtained unambiguously from light scattering data. As is shown in Fig. 1 the majority of the results fall in a somewhat broad band suggesting the existence of some uniform relationship between the size and the shape of the particles. Several preparations, however, differ markedly from the rest being apparently more compact for their size than the others. No complete explanation for these differences has yet been produced, although POUYET et al. (1957) have suggested tentatively that the shape of the particles may be influenced by the use of salt or alcohol during preparation.

Information about the shape of molecules can in theory be obtained from light scattering experiments but in the case of DNA the interpretation of the data for the angular distribution of the scattered light is difficult. Different authors have suggested that the particles are rigid rods (OSTER, 1950), statistical coils (SMITH and SHEFFER, 1950), stiff coils (SMITH and SHEFFER, 1950; ROWEN et al., 1953; ROWEN, 1953; PETERLIN, 1953a; REICHMANN et al., 1954), or branched coils (DOTY and BUNCE, 1952). The more recent data appear to preclude the possibility that the particles are rigid rods and no other evidence in favour of a branched chain molecule has been reported. REICHMAN et al. (1954) have shown that the difference between the observed results and those calculated for a random distribution of statistical coils can be explained either (a) by assuming that the particles are statistical coils with a degree of heterogeneity characterized by $M_w/M_n \approx 6$ or (b) if it is assumed that $M_w/M_n = 2$ then it can be calculated that about half the particles are too stiff to fold into the shape of statistical coils. Attempts have been made to deal theoretically with this problem of the stiff coil and PETERLIN (1953a,b) has developed a theory for a homogeneous system of long chain molecules in which the shape of the molecule is related to the stiffness of the chain by a

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(827)

differences exist between different preparations. The observed heterogeneity appears to rule out the possibility that the DNA particles exist in solution as rigid rods of uniform diameter since PEACOCKE and SCHACHMAN (1954) have shown that for such a system, variations in the length of the particles have very little effect on the sedimentation constant. The particles must therefore exist in some bent or coiled form or as parallel aggregates of rigid rods of differing diameter. The evidence from light scattering studies appears to favour the former. The possibility that aggregation of some form may contribute to the observed heterogeneity or may be responsible for the variations observed between different preparations cannot be overlooked. Experiments have shown that the addition of chelating agents to solutions
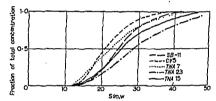

Fig. 3. Sedimentation constant distribution curves for different samples of calf thymus DNA in 0 2 M NaCl extrapolated to infinite dilution.

of DNA do not affect the sedimentation coefficient distribution curves (SHOOTER et al., 1956a). This suggests that aggregation due to the presence of polyvalent metal ions does not occur. Further support for this conclusion was obtained by BUTLER et al. (1954) from measurements of the viscosity of DNA solutions in the presence of many different cations. These authors showed that the observed reduction in viscosity on the addition of a variety of salts could be attributed solely to the change of the ionic strength of the solutions. The possibility that aggregation might occur through hydrogen bonding has been investigated by adding to solutions of DNA large concentrations of urea. The results obtained indicate either that urea has no effect (DOTY and RICE, 1955; ALEXANDER and STACEY, 1955a) or that it separates the twin strands of the helix (see later) (ALEXANDER and STACEY, 1955a,b, 1957; CONWAY, 1956; PAIN, 1956); in neither case has any evidence of the breakdown of aggregates of the twin strand complex been observed. These experiments therefore suggest that aggregation of particles through hydrogen bonding does not occur.

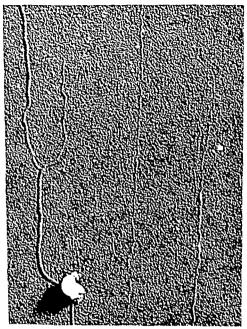It is known that preparations of DNA in general contain small

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Fig. 2. Electron-microscope photograph of DNA. Magnification × 96,000.

1956b, 1957). This suggests that any aggregates present in DNA preparations also occur in the nucleoprotein. Preparations of DNA using the chloroform deproteinization method on the other hand, were found to give a DNA with a markedly different distribution from that observed in the parent nucleoprotein (Fig. 5) suggesting that this method removes protein more efficiently than the detergent method. Since, however, these preparations have been shown to contain aggregates cross-linked by residual protein (BUTLER et al., in press) it would appear that this method of preparation fractionates the DNA much of the material of high sedimentation coefficients being lost in the denatured protein precipitate. It seems likely that the presence of varying amounts of these high molecular weight aggregates in
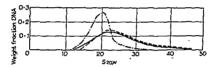

Fig. 5. Sedimentation coefficient distribution curves for calf thymus DNA.
—— nucleoprotein A in 2·5 M NaCl.
– – – DNA prepared from A by detergent method: in 0·2 M NaCl.
—·— DNA prepared from A by Sevag method: in 0·2 M NaCl.

different preparations may be responsible for some of the variations of molecular weight observed in light scattering experiments. It is of particular interest to note that the high molecular weights of some of the samples of DNA reported by SADRON (1955) do not appear to be due to the presence of protein linked aggregates since it was found that the distribution of sedimentation coefficients of one of these preparations, CV5, was not affected by the addition of chymotrypsin.

The results of the sedimentation, light scattering and electron microscopy experiments suggest that DNA exists in solution as a heterogeneous system of bent or coiled molecules. The next problem is to determine whether the observed heterogeneity is due to variations in the size or the shape of the molecules. Electron microscope photographs clearly indicate that variations in the size of the particles occur. If the heterogeneity were to any great extent due to variations in the shape of the particles (e.g. the smaller particles approximating to rigid rods and the larger to flexible coils), altering the shape of the particles by changing the solvent or degrading the molecules should affect the shape of the sedimentation heterogeneity distribution and change the relationship between S or $[\eta]$ and M. Sedimentation experiments using

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amounts of residual protein ($<$ 1 per cent) (KAY *et al.*, 1952). It was found (SHOOTER, 1957; BUTLER *et al.*, in press) that when chymotrypsin was added to a solution of a DNA containing 0·7 per cent of residual protein, a marked change in the sedimentation coefficient distribution curve occurred (Fig. 4). This change was attributed to the breakdown of particles cross-linked by protein. In the example illustrated the proportion of aggregated DNA was found to be 26 per
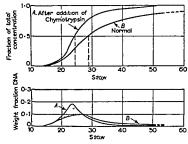

Fig. 4. Sedimentation coefficient distribution curves showing the effect of adding chymotrypsin to a sample of calf thymus DNA containing 0 8 per cent residual protein. Upper figure, integral curves. Lower figure, normal distribution curves; the area of the shaded portion is equal to the fraction of DNA originally present as protein linked aggregates.

cent. Further experiments have shown that the amount of the DNA aggregated decreased as the amount of residual protein decreased. By increasing the number of stages in which the DNA was treated with detergent, it was found possible to reduce the amount of residual protein to less than 0·1 per cent and with these preparations no evidence of aggregation was observed. The elimination of these protein linked aggregates considerably reduced the variations observed between different preparations of DNA from the same source. Even after treatment with chymotrypsin, however, the preparations showed a marked sedimentation heterogeneity. Analysis of the residual protein has shown that it is not a histone nor is it derived from connective tissue (BUTLER *et al.*, in press). Whether or not it is a component of the nucleoprotein complex is not yet known. Experiments have shown that the distribution of sedimentation coefficients of the DNA in the nucleoprotein complex is similar to that observed for the DNA prepared from it using the detergent method (SHOOTER and BUTLER,

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that the proportionality constant k in the equation $S = kM^{0.38}$, is dependent on the degree of polydispersity (DOTY, 1957).

V. THE FLEXIBILITY OF THE DNA MOLECULE

The results of the physical measurements described in the previous sections suggest that DNA molecules in solution are coiled but the degree of flexibility of the particles cannot be unambiguously determined until a more precise measure of the heterogeneity is available. The stiffness of DNA in comparison with other polymer molecules can be exemplified by calculating from the light scattering data the ratio of the mass of the molecule to the volume it occupies assuming that it is a statistical coil. In effect this is equivalent to calculating the concentration of polymer at which free rotation of the molecules becomes possible. For methacrylic acid this concentration is 0·3 per cent, for cellulose derivatives about 0·07 per cent and for DNA about 0·003 per cent. These figures clearly indicate that the DNA molecules have a high modulus of elasticity as might be expected from their twin strand structure.

Attempts have been made to assess the flexibility of the DNA molecule from measurements of the dimensions of the particle in water and in salt solutions. By analogy with the behaviour of other poly-electrolytes, it might be expected that the reduction in the electro-static repulsion between the phosphate groups of DNA on the addition of salt to a solution in water would lead to a general contraction of the molecule. ROWEN et al. (1953) and GEIDUSCHEK (1951) found that the radius of gyration of the particles measured by light scattering was almost 50 per cent less in salt solution than in water. Similar measurements have been made by ALEXANDER and STACEY (1955b) but these authors report that it was not possible to make measurements at sufficiently low concentrations in water for the dimensions of the particle to be established with any degree of certainty. They did, however, observe a small decrease in the radius of gyration on increasing the salt concentration from 0·2 M to 0·9 M, indicative of a limited degree of flexibility. The addition of salt to moderately concentrated solutions of DNA in water has been found to reduce the viscosity considerably and to a large extent to eliminate the non-Newtonian viscosity. This effect was at first attributed (JORDAN, 1951) to a reduction in the asymmetry of the molecules on the addition of salt. Experiments at low concentrations (down to 0·001 per cent) and at low rates of shear have however shown that the intrinsic viscosity is approximately the same in water and salt solutions (POUYET, 1952; CONWAY and BUTLER, 1954). These results indicate that the molecules possess a high degree of rigidity. CONWAY and BUTLER (1954) have suggested that the high viscosity of DNA in water is predominantly

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a series of solutions of DNA degraded to different extents by the action of deoxyribonuclease have shown that the degradation is associated with a progressive movement of the distribution curve to lower values of sedimentation coefficients but the shape of the distribution curve is not affected (SHOOTER, unpublished) (Fig. 6). GEIDUSCHEK (1956) has shown that in alcohol solutions DNA has a much higher sedimentation rate than in salt solution, suggesting that the configuration of the particles is much more compact. SHOOTER (unpublished) has found that
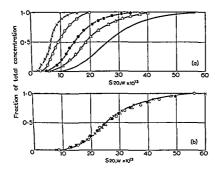

Fig 6 (a). Sedimentation coefficient distribution curves of calf thymus DNA at different times after the addition of deoxyribonuclease. Full line 0 hr, △ 2 hr, ● 5 hr, ○ 23 hr, × 96 hr. (b) Comparison of the shapes of the distribution curves. Full line as in (a). The abscissae of the other curves have been multiplied by (S average)₀ hr/(S. average)ₜ hr Symbols as in (a).

this change in the shape of the molecules does not affect the shape of the distribution curve. DOTY (1957) has recently reported that for a series of degraded samples of DNA, in the molecular weight range 4×10^6 to 3×10^5, $S_0 \alpha M^{0.36}$ and $[\eta] \alpha M^{1.1}$. This evidence therefore strongly suggests that the observed heterogeneity is due to variations in the molecular weight of the particles but this cannot be regarded as completely proved in view of the results reported by SADRON (1955) which were discussed above.

Although the relationship between S and M has been obtained it is not possible to transpose the distribution of g (weight fraction of DNA) against S to distributions of g against M, since it can be shown that the average value of M calculated from the distribution $g = f(s)$ and $g' = f(s^x) = f(M)$ will only be the same if α is unity. It would appear

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PETERLIN (1954) and have concluded that the DNA is intermediate *between a rigid rod and a statistical coil.*

In contrast to the results of the experiments described above, *studies of the variations of the radius of gyration of DNA molecules with pH have shown that a reversible contraction of 30–50 per cent may occur in the pH range 7·0–2·6* (ALEXANDER and STACEY, 1955b; REICHMANN *et al.*, 1953). It is doubtful though whether this degree of flexibility can be attributed to the intact DNA molecule. The ionization of the bases at low pH results in the rupture of the hydrogen bonds holding the twin strands together thereby introducing points of considerable flexibility within the molecule (see section on denaturation).

The work of GEIDUSCHEK (1956) on the *sedimentation characteristics of DNA dissolved in alcohol also shows that the molecules can be deformed to some extent.*

VI. DENATURATION

THOMAS (1953) has shown that the extinction coefficient of DNA at the absorption maximum at 260 mμ is some 40 per cent less than that calculated from the extinction coefficients of individual nucleotides. He has also found that mild treatments, e.g. heating, increasing or decreasing the pH, and decreasing the concentration of Na$^+$ ions (THOMAS, 1951, 1953, 1954), result in irreversible increases in the absorption of DNA solutions at 260 mμ. Since these changes are in general not associated with any change in the molecular weight, THOMAS suggested that they should be denoted as "denaturation" reactions.

Several hypotheses have been put forward to explain the low absorption of solutions of DNA in the region near 260 mμ. THOMAS (1953) attributed the effect to dipole interaction between the bases lying parallel to and on top of each other. LALAND *et al.* (1954) suggested that the effect was due to the overlap of π orbitals of adjacent bases. Recently LAWLEY (1956c) has investigated in detail the absorption spectrum of normal DNA and of the denatured product obtained (a) by heating solutions in the presence of salt and (b) by the addition of 10^{-2} N HCl. The results showed that the shape of the absorption curve was not affected by heat denaturation but that the extinction coefficient increased by the same proportion (e.g. 25 per cent after treatment at 96°) at all wavelengths in the range 230–300 mμ (Fig. 8). The shape of the absorption spectrum on acid-denaturation changed in the way that was expected to accompany the addition of protons to the basic groups of the purines and pyrimidines, but in addition to this there was an overall increase in the extinction coefficients at wavelengths 230–280 mμ of about 25 per cent (Fig. 8). It is thus possible to divide the changes in absorption into two parts; those due

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due to electrostatic interaction between the molecules. Denaturation of the DNA in the absence of salt (see section on denaturation) may however result in a change in shape of the molecule which might obscure changes due to a salt effect.*

Measurements of flow birefringence by HORN *et al.* (1952) showed that the behaviour of DNA is analogous to that of a rigid rod molecule
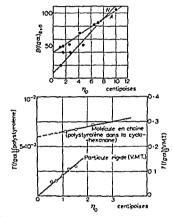

Fig. 7. Curves showing the variation of extinction angle with viscosity of the solvent upper figure for DNA; N at pH 7 and A at pH 3.8, lower figure for tobacco mosaic virus and polystyrene. For rigid particles the curve passes through the origin; for deformable particles the curve has a positive intercept.

such as tobacco mosaic virus (Fig. 7). CERF (1954) has reconsidered the results of this work and has concluded that the DNA molecule can be deformed to some extent. MATHIESON and PORTER (1954) have evaluated the results of their measurements of viscosity and streaming birefringence in terms of the theory of PETERLIN and STUART (1953).

* EDSALL (in press) has studied the variation of the reduced specific viscosity with rate of shear, DNA concentration, and salt concentration. He concludes that the shape of the molecule is not affected by changing the concentration of salt from 1.0-0.10 M. Comparison of the curves showing the effect of rate of shear with those predicted for rod-like systems suggests that the DNA molecules behave as rods with high internal viscosity rather than as rigid particles.

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Considerable support for this proposed mechanism of denaturation is provided by the work of THOMAS and DOTY (1956) on the degradation of DNA at pH 2·6. Three reactions were found to occur simultaneously (a) cleavage of phosphodiester bonds resulting in degradation, (b) cleavage of glycosidic nitrogen bonds to liberate purines, and (c) the cleavage of the hydrogen bonds. At the beginning of the reaction the kinetics followed the course expected for the scission of a twin chain molecule held together at a few points separated by regions, corresponding in length to about 1000 nucleotides, in which the chains were separated. After the molecular weight had fallen to about one half of the initial value the kinetics of the reaction corresponded to the scission of single polymer chains. At this stage of the reaction measurements of viscosity and molecular weight showed that $[\eta] \alpha k M^{\frac{1}{2}}$ indicating that the single polynucleotide chains behave as impermeable, flexible coils in contrast to the extended, free draining character of normal DNA molecules at pH 7·0. The results suggested that at pH 2·6, the majority of the hydrogen bonds were broken very quickly. Whether the remaining junction points were residual hydrogen bonds or regions of chain entanglement could not be decided. Further evidence of the flexibility of the single polynucleotide strands has been provided by the birefringence studies of HORN et al. (1952). At pH 3·8 the behaviour of DNA was found to be analogous to that of a flexible chain polymer such as polystyrene in contrast to the high degree of rigidity observed in solutions at pH 7 (Fig. 7). It has already been mentioned that electrometric titration data suggest that at high and low pH the hydrogen bonds holding together the twin strand structure together are broken and that a more random structure is formed on reneutralization. GULLAND et al. (1947) and more recently COX and PEACOCKE (1956) have shown that the electrometric titration curves from pH 2–12 and from pH 12–2 are identical, i.e. the pairs of bases can be separated either by the addition of protons at low pH or the removal of protons at high pH. The latter authors (COX and PEACOCKE, 1957) have investigated the effects of cycles of titration from pH 7 to lower and lower pH and have obtained evidence of a progressive breakdown of the ordered structure.

Heat denaturation has been studied in many laboratories. The stability of DNA preparations exhibit some variation, but in general it has been found that up to 70–80° they are not affected by heat (ZAMENHOF et al., 1953; AVERY et al., 1944; DOTY and RICE, 1955; COX and PEACOCKE, 1957; ZAMENHOF et al., 1954). At higher temperatures the viscosity of the solutions falls markedly and considerable reduction in the radius of gyration of the particles has been observed. The energy of activation is extremely high (DOTY and RICE, 1955; LAWLEY, 1956) suggesting that the denaturation process

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to chemical changes of the molecule (i.e. titration of the bases) and those which must be due to some irreversible change in the structure of the molecule which is not associated with any chemical change. On the basis of these results LAWLEY has suggested that the low absorption of DNA is due to a geometrical effect, the ordered packing of the pairs of bases within the molecule reducing the total effective absorbing area of the chromophoric groups. If it is assumed that absorption occurs preferentially when the electric vector lies parallel to the planes of the bases then, as COMMONER (1949) has shown, for a fully ordered
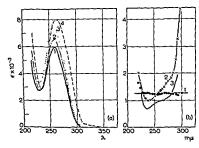

Fig. 8 (a). Ultraviolet absorption spectra of calf thymus DNA: (1) in 0 01 N Na+ phosphate pH 7·2, 20°C; (2) in water 20°C; (3) as (1) heated to 96°C cooled to 20°C; (4) in 10^{-3} N HCl. (b) Ratio of extinction coefficients of denatured and undenatured DNA: (1) heat denatured DNA, as (a 3); (2) DNA at pH2, as (a 4); (3) calculated for addition of acid to pH 2.

structures of this type a decrease in absorption of 50 per cent is to be expected. The somewhat lower reduction (40 per cent) in the case of DNA may be due either to the presence of some disordered structure or to the fact that the bases are not stacked exactly on top of one another but form a spiral within the helix. It should be noted however that it has not yet been proved that absorption in purines and pyrimidines occurs preferentially when the electric vector lies in the planes of these molecules. On the basis of the above hypothesis LAWLEY has suggested that the process of denaturation involves (a) the breaking of the hydrogen bonds between the pairs of bases and separation of sections of the two polynucleotides chains and (b) a rearrangement of the more flexible single chains to configurations in which the purine and pyrimidine rings are no longer superposed with a consequent increase in the absorption.

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the salt concentration reduces the temperature at which a decrease in viscosity (ZAMENHOF et al., 1954) and an increase in E_{260} are observed (LAWLEY, 1956c).

It is of great interest to determine to what extent the separation of the twin chains of the DNA molecule is reversible. The viscosity experiments of HORN et al. (1952) indicated that irreversible changes in the shape of the molecule occurred at pH 3·8. The birefringence experiments of MATHIESON and MATTY (1957) suggested that reversible changes in shape occurred between pH 7 and pH 4·5 but that at lower pH the effects were irreversible. ALEXANDER and STACEY (1955b, 1957) on the other hand found that the reduction in the radius of gyration of the particles observed between pH 7·0 and 3·8 was to a large extent reversible. The latter authors found that after reducing the pH to 2·6 the radius of gyration on reneutralization only returned to about half its original value. REICHMANN, BUNCE, and DOTY (1953) performed similar experiments and observed an almost completely reversible change in shape of the particles after treatment at pH 2·6. In these latter experiments however the changes of pH were obtained by dialysis at 4°. COX and PEACOCKE (1957) have recently found that when a solution of DNA was acidified to pH 2·6 and reneutralized at 4°, on subsequent titration at room temperature the forward titration curve differed from the back titration curve. These results and those of REICHMANN et al. (1953) suggest that at low temperatures the rotation of the free single polynucleotide chains is hindered sufficiently for the hydrogen bonding to be broken to some extent reversibly.

Spectrophotometric titration curves show that on lowering the pH the increase in E_{260} occurs over a quite narrow range of pH and does not appear to be correlated with the electrometric titration curves (BEAVEN et al., 1955). In a recent paper LAWLEY (1956c) has investigated the effects on the absorption spectrum of DNA of lowering and then increasing the pH. As mentioned above, the increase in E_{260} appears to be mainly due to changes in the structure of the molecule whereas the increase of E_{280} is associated with both changes in structure and changes due to the addition of protons to the bases (mainly cytosine with a small contribution from adenine). The ratio E_{280}/E_{260} is therefore a measure of the extent to which the bases are titrated. Some of the results obtained are shown in Fig. 9a and b. After titration from pH 7 to pH 4 or 3 only a small decrease of E_{260} is observed on back titration. In contrast to this the ratio E_{280}/E_{260} returns to its initial value after titration to pH 3 as is to be expected if this measures only the changes due to the addition or removal of protons from the bases. Comparison of the two figures shows that, on lowering the pH, E_{280}/E_{260} increases before any change in E_{260} is observed. This suggests that a considerable proportion of the bases can be titrated before any irreversible changes

requires the cumulative rupture of a series of hydrogen bonds. Measurements of the absorption of solutions that had been heated and then cooled showed increases in E_{260} of 20–24 per cent (THOMAS, 1953; THOMAS, 1951, 1954; LALAND et al., 1954; DEKKER and SCHACHMAN, 1954). More direct evidence of the rupture of hydrogen bonds on heating has been obtained by COX and PEACOCKE (1956) from electrometric titration studies. They found that the difference between the forward and back titration curves to acid pH decreased as the temperature at which the solution had been heated increased. From these curves the fraction of hydrogen bonds broken as a result of heating was calculated and it was found to be linearly related to the observed increase in E_{260}. Similar experiments with DNA which had been denatured and degraded by γ-irradiation (COX and PEACOCKE, 1956) also indicated a linear relation between the increase of E_{260} and the fraction of hydrogen bonds which were broken.

Very little is known as yet about the changes in the molecule that are associated with the denaturation which occurs in solutions of the low salt concentration. SHOOTER and BUTLER (1956a) reported that the distribution of sedimentation coefficients observed in 0·2 M NaCl was the same irrespective of whether the DNA had first been dissolved (at 0·01 per cent) in water or in 0·01 M NaCl. The sedimentation coefficient is, however, probably an insensitive measure of changes in shape of highly asymmetric particles. It may be noted that POUYET et al. (1950) and DHAUSSY (1954, 1955) have observed changes in E_{260} of DNA solutions in water when the concentration is reduced below 0·003 per cent. DHAUSSY (1954, 1955) has reported that a change in dielectric constant also occurred at this concentration and SHOOTER and BUTLER (1956a) found that the average sedimentation coefficient of DNA in water increased rapidly below 0·003 per cent. These effects were attributed to the breakdown of ordered structures but it seems likely that denaturation effects may account for some of the changes observed. At high concentrations of DNA the Na+ associated with it may prevent denaturation. Although no evidence of the breaking of hydrogen bonds under these conditions has been reported it seems probable that this does occur. LAWLEY (1956) has suggested that at low salt concentration Na+ is dissociated from the phosphate groups and that hydrogen bonds are more easily broken as a result of the increased electrostatic separation between the twin chains. Other experiments have shown that reduction of the salt concentration increases the sensitivity of DNA to denaturation. JORDAN et al. (1956) and COX and PEACOCKE (1956b), for example, have found that the electrometric titration curves (acid) are moved to higher values of pH as the ionic strength of the solution is decreased. Spectrophotometric titration curves exhibit similar effects (LAWLEY, 1956c). Reducing

is about three times as high as that observed in water alone (Fig. 10). This increase was attributed to the separation of the *twin chains of the molecules* to give twice the number of hydrodynamic units of approximately the same degree of asymmetry as the original molecules. The greater flexibility of the denatured product was demonstrated by the fact that the addition of salt reduced the intrinsic viscosity considerably (Fig. 10). These results have been corroborated by PAIN (1956). DOTY and RICE (1955) have found that the addition of 4 M urea to a solution of DNA in the presence of NaCl reduced the temperature at which the marked fall in viscosity occurred by 17°C. Since a partial
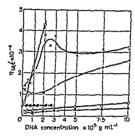

Fig. 10. Effect of urea on the reduced specific viscosities of DNA.
  ○ in water
  + in 6 M urea before dialysis
  × in water after removing urea by dialysis
  ● in 0 1 M NaCl
  △ in 0 1 M NaCl, 6 M urea before dialysis
  ▲ in 0 1 M NaCl after removal of urea by dialysis.

separation of the twin chains is to be expected below the initial temperature these experiments and those of CONWAY and BUTLER suggest that the addition of urea to partially denatured DNA greatly facilitates further denaturation.

ALEXANDER and STACEY (1955b, 1957) have reported that when 4 M urea was added to a solution of herring sperm DNA in the presence of NaCl at room temperature, the molecular weight fell from 5·9 × 10⁶ to 2·7 × 10⁶. They suggested that this indicated the complete separation of the twin strands of the helix. Electron microscope photographs clearly showed that treatment with urea altered the shape of the molecules profoundly. Similar experiments with calf thymus DNA (ALEXANDER and STACEY, 1955a) on the other hand showed that the addition of urea did not affect the molecular weight. After pretreatment

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in structure occur. After titration to a pH at which an increase in E_{260} is observed, on back titration the curve E_{280}/E_{260} is displaced towards higher pH in a manner analogous to that observed in electrometric titrations. The results suggest that denaturation is reversible provided that the hydrogen bonding is not broken and the two polynucleotide chains are not separated over too great a length.
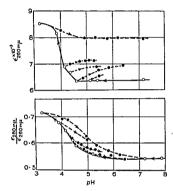
Fig. 9. Changes in absorption of a solution of calf thymus DNA on forward and back titration with acid. The arrow on the curve indicates the direction of the titration.

VII. THE EFFECT OF UREA

A further type of denaturation that has been studied is that produced by the addition of urea to DNA solutions. The investigations can be divided into two groups those involving conditions under which, in the absence of urea (a) the DNA is known to be stable and (b) a partial denaturation of the DNA might be expected. The latter class includes the experiments in which urea is added to DNA solutions in water. CONWAY and BUTLER (1952) have shown for example that the viscosity of solutions of calf thymus DNA in water was reduced by the addition of urea, the extent of the reduction increasing with increasing urea concentration. A further reduction in viscosity was observed when the urea was removed by dialysis. CONWAY (1956) has recently reported that at zero rates of shear the intrinsic viscosity in the presence of 6 M urea

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(Fig. 11). This is to be expected on the basis of the twin strand structure since the probability of a break occurring in one chain close to a break already present in the second chain will initially be low but will increase progressively as the reaction proceeds. THOMAS (1956) has investigated the enzyme degradation in detail, making simultaneous measurements of molecular weight and viscosity. At the same time the extent of the reaction was determined by a titration method. The results obtained parallel those of SCHACHMAN and ZAMENHOF. Analysis of the light scattering data at different stages of the reaction showed that the ratio of the radius of gyration of the particles to their molecular weight decreased by about 40 per cent during the reaction, in contrast to the
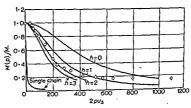

Fig. 12. The rate of degradation of DNA by deoxyribonuclease. Full lines theoretical curves. ⬦ experimental results The abscissae are a measure of the extent of the reaction in terms of the number of phosphodiester bonds broken by the enzyme. The ordinate is the ratio of the molecular weights of the degraded and undegraded DNA.

decrease of 50 per cent which is immediately observed when a solution is acidified to pH 2·6. These results, indicating that the flexibility of the particles is little affected, suggest that in the initial stages of the enzymic degradation the twin chains are not separated. Similar experiments by REICHMANN (1956) have corroborated these results. THOMAS has calculated the relationship between the fall in molecular weight and the extent of the enzymatic reaction for the degradation by random bond breaking of single and double chain structures. Comparison of the curve marked "single chain" with the experimental points in Fig. 12 clearly shows that the results do not correspond to the degradation of a single polymer chain. In the double chain structure, the strength of the hydrogen bonds between the two chains will determine how close together breaks on opposite chains have to be to produce degradation. In Fig. 12 the curves calculated for separations of ($h =$) 0, 1, 2, and 3 pairs of nucleotides are given. The experimental points approximate to the curve for $h = 2$ suggesting that degradation

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of the DNA with ethylenediaminetetracetic acid however, urea reduced the molecular weight from 6×10^6 to 3×10^6. DOTY and RICE (1955) reported that the viscosity of solutions of calf thymus DNA in salt were not affected by the addition of either 4 M or 8 M urea. The results of these experiments in which urea was added to a solution of DNA in which no partial denaturation should have occurred are somewhat conflicting. It would appear from the results with herring sperm DNA that urea can denature DNA without the necessity for preliminary partial denaturation by other means. In the case of calf thymus DNA it seems that other factors not yet known, may affect the stability of the structure.

VIII. DEGRADATION

The degradation of DNA by acid treatment has already been discussed. It was shown that in this system the twin strands of the helix were to a large degree separated before degradation had proceeded very far. When however DNA is degraded by the action of deoxyribonuclease
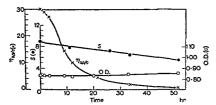
Fig 11. Curves showing the variation with time of sedimentation coefficient, reduced specific viscosity and optical density at 260 mμ of a solution of DNA after the addition of deoxyribonuclease

(LALAND et al., 1954; GOLDSTEIN and STERN, 1950, DEKKER and SCHACHMAN, 1954; KUNITZ, 1950), by ultrasonics (LALAND et al., 1954; GOLDSTEIN and STERN, 1950) or X-ray irradiation (Cox et al., 1955) it has been found that very considerable decreases in the viscosity of the solution occur before any increase in the absorption at 260 mμ is observed. This suggests that in these systems the twin strand structure remains intact until a considerable reduction in the molecular weight has occurred. ZAMENHOF et al. (1954) and DEKKER and SCHACHMAN (1954) have measured the viscosity of a solution of DNA in the presence of a very low concentration of deoxyribonuclease and have shown that the rate of degradation is approximately zero to begin with and accelerates as the reaction proceeds

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electrometric titration data (Cox and PEACOCKE, 1956b) and from a study of the kinetics of enzyme (THOMAS, 1956) and acid degradation (THOMAS and DOTY, 1956). The results suggest that there are few if any of these breaks.

The degradation of DNA by X-rays has been intensively investigated. Work in this field up to 1954 has been reviewed by DAVISON, CONWAY, and BUTLER (1954). The irradiation of solutions of DNA results in an initial fall in viscosity followed by a progressive decrease in viscosity which may continue for many hours (DAVISON et al., 1954). It has been suggested that this "after effect" of the irradiation is due partly to the H_2O_2 formed and partly to the slow decomposition of peroxides or hydroperoxides which result from the interaction of radicals with the DNA molecule (BUTLER and CONWAY, 1950; CONWAY, 1954; CONWAY and BUTLER, 1953; SCHOLES and WEISS, 1950, 1952). SCHOLES, WEISS, and WHEELER (1956) have estimated the amount of peroxide formed as a result of the irradiation of solutions of calf thymus DNA and of yeast RNA in the presence of oxygen by two methods (a) the formation of a coloured complex with titanium sulphate reagent, and (b) the oxidation of KI and the estimation of the amount of iodine liberated. It was found that the amount of peroxide estimated by method (b) was always greater than that estimated by method (a). They attributed the difference observed to the presence of hydroperoxides since it is known that these compounds do not form coloured complexes with the titanium sulphate reagent but do liberate iodine from KI. It was further proved that the concentration of the hydroperoxide decreased progressively after the irradiation; the kinetics of this reaction indicated the presence of two of these derivatives of different stability. Further work showed that hydroperoxide formation occurred mainly with the pyrimidines rather than the purines. Earlier work had demonstrated that the pyrimidines are more susceptible to attack by radicals than the purines (SCHOLES and WEISS, 1950, 1952).

Further work on the degradation of DNA by ionizing radiations has been concerned with the changes in the structure of the molecule which are involved. ALEXANDER and STACEY (1956) have investigated the effects of irradiating herring sperm DNA samples containing 10–15 per cent of water with γ rays and 1·2 MeV electrons at high dose rates. Measurements of the molecular weight of the irradiated samples using light scattering methods showed that the same amount of degradation was produced by equal doses of γ rays and electrons (Fig. 13). The molecular weight falls for doses up to 10^6 r and begins to increase again after higher doses. These results are to some extent confirmed by those of SHOOTER et al. (1956) who have investigated the effect of irradiating samples of calf thymus DNA containing 20 per cent of water with 1·2 MeV electrons. They found that doses of $10^5–10^6$ r caused only

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occurs when bonds are broken on opposite chains not more than two nucleotides apart.

A problem of considerable importance in relation to the structure of the DNA molecule was raised by some experiments reported by DEKKER and SCHACHMAN (1954). These authors observed that when a solution of DNA in water was heated at 100°C, in addition to the denaturation discussed above, a considerable decrease in molecular weight occurred. This latter effect was attributed to the presence of broken bonds in the single polynucleotide chains. Similar experiments in other laboratories have confirmed that DNA is degraded when heated in water but it has been found that the presence of salt in the solution reduces the sensitivity to heat. SADRON (1955), for example, found that after heating a 0·02 per cent solution of DNA for 15 min at 100°C the molecular weight, as determined by light scattering, was reduced by a factor of 20 for solutions in water, and by 2–3 for solutions in 0·2 M NaCl. In the presence of M NaCl no change in molecular weight was observed. The effects of heating 0·01 per cent solutions of DNA in 0·2 M NaCl have also been investigated by SHOOTER et al. (1956) who observed decreases in molecular weight by a factor of 4–8. ALEXANDER and STACEY (1955a) and DOTY and RICE (1955) on the other hand observed no change in molecular weight on heating solutions in 0·2 M NaCl. It would appear that the differences in these results are to be attributed to the effects of heating DNA solutions at different concentrations. SHOOTER et al. (1956) for instance observed that on heating at 0·01 per cent the average sedimentation was unaffected but heating at 0·05 per cent followed by dilution to 0·01 per cent resulted in an increase in the sedimentation coefficient suggesting a smaller reduction in molecular weight. These latter authors also demonstrated that the production of artificial breaks in the molecule by X-irradiation increased the sensitivity to heat. Breaks produced in the single polynucleotide chains by deoxyribonuclease also increase the sensitivity to heat (ZAMENHOF et al., 1954a). If solutions in water are heated for longer times a progressive degradation is observed suggesting that phosphodiester bonds are hydrolysed. If solutions in 0·2 M NaCl are heated for long periods further reduction in the sedimentation coefficient is also observed but the rate of degradation is slow compared with the initial fall in molecular weight. Whether the initial rapid fall in molecular weight indicates the existence of breaks in the single chains cannot be determined with certainty. SHOOTER and BUTLER (1956b, 1957) have recently shown that DNA preparations are probably subject to some degradation by deoxyribonuclease during the isolation of the nucleoprotein from the tissue. Any breaks that are found in the chains are therefore probably artefacts produced during the preparation of the DNA. Attempts have been made to detect the presence of breaks from

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STACEY (1956). They found that the addition of 4 M urea to solutions of the irradiated samples to break hydrogen bonds holding the fragments together, resulted in very marked decreases in molecular weight, e.g. for a dose of 1.8×10^5 r the molecular weight measured in salt solution decreased by only 20 per cent but on the addition of urea the molecular weight was reduced by a factor of about 4 (Fig. 13). They calculated from these latter results that the energy required to produce a single break was between 10 and 20 eV. This represents a highly efficient reaction and they suggested that some of the energy absorbed
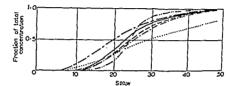

Fig. 14. Sedimentation coefficient distribution curves of DNA.
— · — · — unirradiated DNA
—— 10^5 r
—— — 2×10^5 r
— · — 5×10^5 r
· · · · · · 10^6 r
— — — — 10^5 r after 15 min at 100°C.

by the molecule may be transferred from the initial site of the absorption along the molecule to more labile bonds before chemical reaction occurs.

Further support for the general mechanism of the degradation by ionizing radiations has been provided by the work of Cox *et al.* (1955) on the effects of the γ-irradiation of solutions of herring sperm DNA. These authors have shown by titration studies that the hydrogen bonding between the twin polynucleotide chains is broken to a greater extent the higher the radiation dose. Viscosity measurements on solutions of irradiated DNA in 0·1 M NaCl were made using Ubbelohde viscometers and were found to obey the equation $\eta_{sp}/c = [\eta] + k'[\eta]^2 c$ where c is the concentration and $k' = 0.77$. The latter figure can be compared with the theoretical values of 0·77 for extended chains and 0·73 for rigid rods. Analysis of the variation of the intrinsic viscosity with radiation dose and DNA concentration following the theory of CHARLESBY but taking into account the twin strand structure of the molecule showed that the molecular weight and viscosity were related by the equation $[\eta] = kM^{0.93}$. This suggests that the structure of the molecule is intermediate between that of a rigid rod ($[\eta] = kM^{1.7}$) and

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small changes in viscosity as measured in a Frampton viscometer. At doses of 10^5 r the average sedimentation coefficient is not greatly affected but there is a marked increase in heterogeneity resulting from an increase of material with both higher and lower sedimentation coefficients (Fig. 14). These results suggest that irradiation under these conditions produces a combination of degradation and aggregation. At doses of 10^6 r aggregation predominates. KOENIG and PERRINGS (1953) also found that X-irradiation of vacuum dried DNA produced a
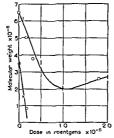

Fig. 13. The decrease in the weight average molecular weight of DNA on irradiation. Upper curve, molecular weight measured in 0.2 M NaCl; lower curve, measured in 0.2 M NaCl + 4 M urea
△ and ○ γ rays (1200 r/min)
| and × 1.2 MeV electrons (9 × 10⁵ r/min)

small increase of the sedimentation constant (extrapolated to $c = 0$ from measurements in range 1 per cent to 0·025 per cent) after a dose of 10^6 r though a decrease was observed after a dose of 10^7 r. Further evidence of aggregation was observed by SETTON and DOYLE (1954) who found that after irradiating dry DNA with high doses (5×10^6 rep) of electrons or deuterons it was soluble in water and formed gels in 0·2 M NaCl. These authors also found that dry DNA irradiated with ultraviolet light (185–300 mμ) formed gels on the addition of water. Since on irradiation with γ-rays bonds are broken at random it is to be expected that some breaks will occur in the single polynucleotide chains opposite to intact portions of the second chain and will not therefore result in a degradation of the molecule as a whole. The existence of such breaks can be inferred from the observation that as the dose increases the sensitivity of the DNA to heat degradation also increases (SHOOTER et al., 1956). A more direct demonstration of the existence of these breaks has been obtained by ALEXANDER and

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on forward titration. The extra bases that can be liberated by further denaturation at pH 3 (QR) is thus reduced. Comparison of the curves showing the difference between forward and back titration as a function of pH shows that the bases liberated by heat denaturation titrate over the whole pH range from 3·1 up to 7·0. In contrast to this the difference curves obtained from DNA denatured by γ-irradiation (Fig. 16) show that bases titrating in the lower part of the pH range, i.e. adenine, are liberated first and that groups titrating nearer to pH 7, i.e. cytosine, are only liberated after more extensive irradiation.
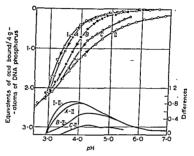

Fig 16 Titration curves of herring sperm DNA. Upper figure (ı) and (ıı) forward and back titration curves of unirradiated DNA; A, B, C forward titration curves after increasing doses of γ-rays. Lower figure, difference curves, obtained by subtracting the ordinates of curve (ıı) from the other curves

IX. THE INTERACTION OF DNA AND METAL CATIONS

CREETH and JORDAN (1949) have calculated the charge on the DNA molecule in the presence of various concentrations of NaCl from measurements of membrane potentials and have found that the charge is only $\frac{1}{4}$ to $\frac{1}{2}$ of that expected. This difference has been attributed to the binding of Na+ ions to the phosphate groups of the DNA. Similar measurements by SHACK et al. (1952) have confirmed these observations though it was found that the increase in the negative charge on the molecule with increasing concentration of NaCl was not as great as that reported by CREETH and JORDAN. Distribution measurements suggested that at higher concentrations of NaCl, Cl⁻ ions were also bound to the molecule thus explaining the observed increase in negative charge. SHACK et al. (1953) have obtained further confirmation of the binding of Na+ from studies of the decrease in the absorption

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of a random coil ($[\eta] = kM^{0\cdot 66}$). The observed dependence of $[\eta]$ on M is in good agreement with that obtained by BUNCE and RICE (see DOTY, 1957) from viscosity measurements on a series of samples of DNA degraded by ultrasonics, $[\eta] = kM^{1\cdot 1}$.

An interesting feature associated with the degradation of DNA by γ-rays has been reported by Cox *et al.* (1955). Electrometric titration experiments with degraded samples have shown that the hydrogen bonds between the pairs of bases adenine and thymine appear to be
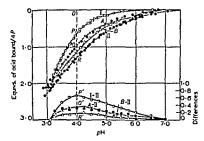

Fig. 15. Titration curves of herring sperm DNA. Upper figure (i) and (ii) forward and back titration curves of normal DNA. Other curves after heating for 1 hr at: ○70°C, ○ 75°C, ×78 8°C, ● 83 4°C, △ 87 5°C, small full circles 100°C and back titration curves of all heated samples. Lower figure, difference curves obtained by subtracting the ordinates of curve (ii) from the other curves.

broken more quickly than those between guanine and cytosine. This is the opposite to the effect observed on acid denaturation where their results suggest that the bonds between cytosine and guanine are broken preferentially (Cox and PEACOCKE, 1957) (The results of JORDAN *et al.* (1956) on the other hand suggest that on acid denaturation the bonds between the two pairs of bases are broken at the same pH.) Similar titration experiments with DNA degraded by heating (Cox and PEACOCKE, 1956a) or by ultrasonics (Cox and PEACOCKE, 1957) showed that under these conditions the bonds between the two pairs of bases were broken with equal facility The results obtained by Cox *et al.* for the effects of heat and γ-irradiation are illustrated in Figs. 15 and 16. In Fig. 15 the difference between the forward and backward titration curves at any pH (e g. *PR*) represents the increase in the number of groups titrating at this pH which are liberated by acid denaturation at pH 3. Partial denaturation by heating liberates some of the bases, as represented by *PQ* for curve *A*, which can add protons

DNA to which the dye molecules can be bound. It was suggested that these sites were the monoesterified and the diesterified phosphate groups. The existence of such a large fraction of monoesterified phosphate groups as is suggested by these experiments ($\frac{1}{15}-\frac{1}{10}$) would suggest either that the molecule is highly branched or that many breaks occur in the single strands as proposed by DEKKER and SCHACHMAN (1954). No other evidence in favour of a branched-chain molecule has been produced and as discussed above, in the majority of preparations, few if any breaks occur in the single polynucleotide chains. The proportion of monoesterified groups estimated in this way varies widely and the changes observed when the pH is lowered are contrary to those that would be anticipated (PEACOCKE, 1955). Recent work by IRWIN and IRWIN (1954) on the interaction of DNA with a 9-amino-acridine derivative and of LAWLEY (1956b) on the interaction of DNA with 5-amino-acridine and with rosaniline has shown that the results can best be described in terms of the existence of one type of binding site only. PEACOCKE and SKERRETT (1956) have investigated the interaction with proflavine (2:8 diamino-acridine sulphate) and have deduced from the results that the sites on the DNA molecule available for absorption exhibit a wide range of affinities for these molecules. There is however evidence that aggregation of the proflavine occurs at the higher concentrations used and the apparent variations in the binding affinity can be best described in terms of a strong, specific binding of single proflavine molecules and a much weaker binding of the aggregates.

Comparative studies on the strength of the binding of different organic cations to DNA have been made by MORTIMAND et al. (1954) and by LAWLEY (1956b). The former authors obtained evidence of a

[several illegible lines]   during

phosphate groups with these particular molecules. LAWLEY (1956b) has found that 5-amino-acridine has a greater affinity for DNA than has rosaniline. Consideration of the structure of rosaniline suggests that bonding to only one phosphate group can occur. The extent to which rosaniline and 5-amino-acridine are bound to DNA was found to be considerably reduced if the DNA was denatured by heat treatment. KURNICK (1954a,b) has found that the affinity of DNA for the dye methyl green and its susceptibility to the attack of deoxyribonuclease are also considerably reduced by heat denaturation. It would appear, as LAWLEY suggests, that in the more random structure produced by denaturation the phosphate groups are to some extent blocked by the formation · · · · · · bonds. It was however found that Na+ ·   ·   ·   ·   rosaniline or 5-amino acridine from denatured than from normal DNA. LAWLEY has suggested that

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at 260 mμ observed on the addition of NaCl to DNA solutions in water.
Similar experiments with Mg^{2+}, Ba^{2+}, and Ca^{2+} showed that these ions
are bound much more effectively by the phosphate groups than is
Na^+. The results suggested that a tight binding of 1·6 atoms of divalent
cation per 4 phosphate groups. This latter result has been confirmed
by measurements of the conductivity of DNA solutions containing
different concentrations of M^{2+}. Further experiments have suggested
that the extent of the binding of Na^+ is about the same for normal and
denatured DNA.

JORDAN and his colleagues have approached this problem from
another angle. From a series of electrometric titration curves at
different ionic strengths and different concentrations of DNA (JORDAN
et al., 1956) they have calculated the number of protons added to the
molecule under different conditions. Assuming that between pH 3 and
7 all the phosphate groups carry a single negative charge they have
been able to calculate the net negative charge on the molecule. From
electrophoresis experiments (MATHIESON and McLAREN, 1956) under
similar conditions the effective charge on the molecule (i.e. including
any Na^+ or Cl^- ions bound to the DNA) has been calculated. The values
of the effective charge at different ionic strengths were comparable
with those obtained by CREETH and JORDAN and by SHACK et al.
Comparison of the titration and mobility data indicated that Na^+ ions
were bound to up to 90 per cent of the phosphate groups. It was
further deduced that a considerable proportion of Cl^- ions are bound
to DNA at low pH. Cox and PEACOCKE (1956b) have analysed their
results on the effect of ionic strength on the titration curves in terms
of several models in an attempt to distinguish between (a) a binding
of Na^+ to DNA and (b) no binding of Na^+ but a greater shielding of the
ionic atmosphere around the DNA particles. Their results are some-
what inconclusive though the evidence favours the binding of the Na^+
ions.

X. THE INTERACTION OF DNA AND ORGANIC CATIONS

Studies of the interaction of various cationic organic compounds with
DNA have suggested that the sites of absorption available to these
molecules are the same as those to which metal cations are bound
(CAVALIERI et al., 1950, 1951, IRVIN and IRVIN, 1954; LAWLEY,
1956a,b). In agreement with the results on the relative strength of
binding of monovalent and divalent cations discussed above it has
been found that the latter are much more effective in displacing
bound organic cations than the former (LAWLEY, 1956a,b). Work
by CAVALIERI et al. (1950, 1951) on the interaction of DNA with
rosaniline suggested that there are two sites of different affinity in

the formation of an NH_3^+ group. It is clear that this problem requires further consideration.

The work of JORDAN and his colleagues (1956), Cox and PEACOCKE (1956a), CAVALIERI and STONE (1955) and LEE and PEACOCKE (1953), has shown that the apparent dissociation constants of the bases in denatured DNA increase as the ionic strength of the solution is reduced. It does however appear to be possible to assign apparent dissociation constants to the bases under specific conditions and from the known composition of the DNA, to calculate a theoretical titration curve which agrees well with that observed except in the pH range 6–8·5 (GULLAND et al., 1947; JORDAN et al., 1956; PEACOCKE, 1954, 1955). The experimental curve indicates that groups are present which titrate in this range. Since the highest pKa of the bases, that of cytosine ($pKa = 4·6$), is far removed from this region it has been suggested that the difference between the theoretical and experimental curves could be attributed to the presence of a small proportion of mono-esterified phosphate groups with a pKa in the range 6·4–6·6 (GULLAND et al., 1947; JORDAN et al., 1956; PEACOCKE, 1954, 1955). Recent experiments with apurinic acid have however shown that the pKa of cytosine in this compound is 5·0 (HURLEN et al., in press). Using this new value Cox and PEACOCKE (1956b) have obtained good agreement between theoretical and observed titration curves of DNA without the necessity for postulating the presence of monoesterified phosphate groups.

It may be noted that in the calculations discussed above it has been assumed that the pKa of the bases is not affected by variations in the charge on the DNA molecule which result from changes of pH. The validity of this assumption has been questioned (DOTY and RICE, 1955). Work on other polyelectrolytes has shown that changes of electrostatic potential resulting from ionization greatly affect the dissociation constants of the ionizing groups. KATCHALSKY et al. (1954) have shown that for such systems, dissociation follows the equation

$$pH = pK - \log \frac{\alpha}{1-\alpha} - 0·4343 \frac{e\psi}{kT}$$

where α is the degree of dissociation and the last term gives the correction due to changes in the electrostatic potential, ψ. MATHIESON and McLAREN (1956) have calculated ψ for DNA from electrophoretic mobility data and have shown that the term $0·4343\, e\psi/kT$ changes from 1·14 at pH 3·0 to 1·63 at pH 7. This result is corroborated by the observation of Cox and PEACOCKE (1956b) that the pK of cytosine in apurinic acid was changed by less than 0·25 units in the pH range 3·5–6·5. This small change in the electrostatic potential term with pH explains the absence of spreading of the titration curves and the

this may be due to the binding of the dyes on the planar rings of the bases.

XI. THE INTERACTION OF DNA WITH HISTONES

Much of the work on the association of histones and DNA in the nucleoprotein complex has been reviewed by DAVISON, CONWAY, and BUTLER (1951). It has been found that the degree of dissociation of the nucleoprotein complex increases with increasing salt concentration (CRAMPTON *et al.*, 1954; BUTLER *et al.*, 1953). The base composition of the DNA extracted using different concentrations of salt does not differ (LUCY and BUTLER, 1954). CHARGAFF and his colleagues (CHARGAFF and DAVIDSON, 1955) have however shown that fractions of DNA of different base compositions can be obtained by extracting with increasing concentrations of salt a preparation of nucleoprotein which has been denatured by chloroform treatment. BROWN and WATSON (1953) have investigated the elution of a sample of DNA from a column of Kieselguhr coated with histone using salt solution of continuously increasing strength. They found that a fractionation of the DNA was only achieved when the histone used was first denatured by heat treatment. LUCY and BUTLER (1954, 1955) have shown that the fractionation of the DNA does not necessarily require variations in the salt concentration since DNA and histone fractions of differing composition can be obtained by repeated extraction of a chloroform denatured nucleoprotein with 0·6 M NaCl. The origin of these differences in the binding between histone and DNA molecules of different base composition is still far from clear. Sedimentation analysis of a series of DNA fractions (LUCY, 1956) revealed that the distributions of sedimentation coefficients did not differ very greatly. There is therefore no evidence that the fractionation is a purely physical one, e.g. small molecules being extracted preferentially. It seems likely that binding of histone to sites other than the phosphate groups (e.g. the free amino groups of guanine) may be involved as suggested by BROWN and WATSON (1953).

XII. ELECTROMETRIC TITRATION EXPERIMENTS

Much of the work on the titration of DNA has been referred to in previous sections of this review but a few points remain to be discussed. Several authors have suggested that the titration of the bases involves the addition or removal of protons from groups which are involved in the hydrogen bonding. In some instances this would involve a marked change in the resonance structure of the base. GULLAND (1947), for example, has concluded from spectrophotometric studies that the addition of a proton to N-heterocyclic denaturation does not lead to

REFERENCES

BROWN G. L. and WATSON M. (1953) *Nature, Lond.* **172**, 339.

BROWN G. L., M'EWEN M. B., and PRATT M. I. (1955) *Nature, Lond.* **176**, 161.

BUTLER J. A. V. and CONWAY B. E. (1950) *J. Chem. Soc.* 3418.

BUTLER J. A. V., DAVISON P. F., and JAMES D. W. F. (1953) *Proc. Biochem. Soc.* **54**, xxi.

BUTLER J. A. V. and DAVISON P. F. *Advanc. Enzymol.* (in press).

BUTLER J. A. V., PHILLIPS D. M. P., and SHOOTER K. V. (in press).

BUTLER J. A. V., CONWAY B. E., and JAMES D. W. F. (1954) *Trans. Faraday Soc.* **50**, 612.

CARLISLE C. H. and BERNAL C. H. (1955) *Ann. Rep. Chem. Soc.* **52**, 380.

CAVALIERI L. F., KERR S. E., and ANGELOS A. (1950) *J. Amer. Chem. Soc.* **72**, 4680; (1951) **73**, 2507.

CAVALIERI L. F., ANGELOS A., and BALIS M. E. (1951) *J. Amer. Chem. Soc.* **73**, 4902.

CAVALIERI L. F. and STONE A. L. (1955) *J. Amer. Chem. Soc.* **77**, 6499.

CERF R. (1951) *J. Polymer Sci.* **12**, 35.

CHARGAFF E., CRAMPTON C. F., and LIPSCHITZ R. (1953) *Nature, Lond.* **172**, 289.

CHARGAFF E. and DAVIDSON J. M. (1955) *The Nucleic Acids*, Vols. 1 and 2. New York: Academic Press.

CHARLESBY A. (1954) *Proc. Roy. Soc.* A224, 120; (1955) *J. Polymer Sci.* **15**, 263.

COMMONER B. (1949) *Science* **110**, 31, 41.

CONWAY B. E. and BUTLER J. A. V. (1952) *J. Chem. Soc.* 3075.

CONWAY B. E. and BUTLER J. A. V. (1953) *Trans. Faraday Soc.* **49**, 327.

CONWAY B. E. (1954) *Brit. J. Radiol.* **27**, 42.

CONWAY B. E. and BUTLER J. A. V. (1954) *J. Polymer Sci.* **12**, 199.

CONWAY B. E. (1956) *J. Polymer Sci.* **20**, 299. See also BUTLER J. A. V. (1955) *Aspects Généraux de la Science des Macromolecules* (Editions du Centre de la Recherche Scientifique), p. 153.

COSGROVE D. J. and JORDAN D. O. (1949) *J. Chem. Soc.* 1413.

COX R. A., OVEREND W. G., PEACOCKE A. R., and WILSON S. (1955) *Nature, Lond.* **176**, 919.

COX R. A. and PEACOCKE A. R. (1956a) *J. Chem. Soc.* 2646.

COX R. A. and PEACOCKE A. R. (1956b) *J. Chem. Soc.* 2499.

COX R. A. and PEACOCKE A. R. (1957) *J. Polymer Sci.* **23**, 765.

CRAMPTON C. F., LIPSCHITZ R., and CHARGAFF E. (1954) *J. Biol. Chem.* **206**, 499.

CREETH J. M. and JORDAN D. O. (1949) *J. Chem. Soc.*, 1409.

DANIELS M., SCHOLES G., WEISS J., and WHEELER C. M. (1957) *J. Chem. Soc.*, 226.

DAVISON P. F., CONWAY B. E., and BUTLER J. A. V. (1954) *Progr. Biophys. Biophys. Chem.* **4**, 148.

DERKER C. A. and SCHACHMAN H. K. (1954) *Proc. Nat. Acad. Sci., Wash.* **40**, 894.

DRAUSSY M. (1954) *C. R. Acad. Sci., Paris* **239**, 114; (1955) *J. Chim. Phys.* **52**, 150.

DOTY P. and BUNCE B. H. (1952) *J. Amer. Chem. Soc.* **74**, 5029.

DOTY P. (1955) *Proc. Third Int. Congress Biochem.*, Brussels, p. 135.

DOTY P. and RICE S. A. (1955) *Biochim. Biophys. Acta* **16**, 446.

DOTY P. (1957) *Trans. Faraday Soc.* **53**, 251.

FEUGHELMAN M., LANGRIDGE R., SEEDS W. E., STOKES A. R., WILSON H. R., HOOPER C. W., WILKINS M. F. H., BARCLAY R. K., and HAMILTON L. D. (1955) *Nature, Lond.* **175**, 834.

FLUKE D., DREW R., and POLLARD E. (1952) *Proc. Nat. Acad. Sci., Wash.* **38**, 180.

FRANKLIN R. E. and GOSLING R. G. (1953a) *Acta Cryst.* **6**, 673.

FRANKLIN R. E. and GOSLING R. G. (1953b) *Nature, Lond.* **171**, 740.

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agreement observed between experimental curves and those calculated without considering this factor. The magnitude of this term accounts well for the change in the titration curves with variations of DNA concentration and ionic strength (MATHIESON and McLAREN, 1956).

The figures are taken from the following sources:

Fig. 3. SHOOTER and BUTLER (1956) *Trans. Faraday Soc.* **52**, 734. Fig. 7.

Fig. 4. SHOOTER and BUTLER (in press).

Fig. 5. SHOOTER and BUTLER (1956) *Nature, Lond.* **177**, 1033. Fig. 1(b).

Fig. 7. HORN, LERAY, POUYET, and SADRON (1952) *J. Polymer Sci.* **9**, 531 Figs. 40 and 5.

Fig. 8. LAWLEY (1956) *Biochim. Biophys. Acta* **21**, 481. Fig. 1.

Fig. 9 *idem ibid* part of Fig. 5.

Fig. 10. CONWAY (1956) *J. Polymer Sci.* **20**, 299. Fig. 1.

Fig. 11. DEKKER and SCHACKMAN (1951) *Proc. Nat. Acad. Sci., Wash.* **40**, 894. Fig. 5.

Fig. 12. THOMAS (1956) *J. Amer. Chem. Soc.* **78**, 1861. Fig. 5.

Fig. 13. ALEXANDER and STACEY (1956) *Progress in Radiobiology*, p. 105. Fig. 1.

Fig. 14. SHOOTER, PAIN, and BUTLER (1956) *Biochim. Biophys. Acta* **20**, 497. Fig. 2.

Fig. 15. COX and PEACOCKE (1956) *J. Chem. Soc.* 2646. Fig. 1.

Fig. 16. COX, OVEREND, PEACOCKE, and WILSON (1956) *Nature, Lond.* **176**, 919. Fig. 3.

REFERENCES

ALEXANDER P. and STACEY K. A. (1955a) *Nature, Lond.* **176**, 162.

ALEXANDER P. and STACEY K. A. (1955b) *Biochem. J.* **60**, 194.

ALEXANDER P. and STACEY K. A. (1956) *Progress in Radiobiology*, p. 105.

ALEXANDER P. and STACEY K. A. (1957) *Trans. Faraday Soc.* **53**, 251.

ALLFREY V. G., MIRSKY A. E., and STERN H. (1955) *Advanc. Enzymol.* **16**, 411.

ARTHUR W. T. (1947) *Symp. Soc. Exp. Biol.* **1**, 66.

AVERY O. T., MACLEOD C. M., and McCARTY M. (1944) *J. Exp. Med.* **79**, 137.

BARNE E., JOLY M., and SHOOTER K V (in press).

BAYLEY S T. (1951) *Nature, Lond.* **163**, 470.

BEAVEN G H , HOLIDAY E. R., and JOHNSON E. A ; see CHARGAFF and DAVIDSON (1955) Vol. 1, p. 522.

REFERENCES

PAULING L. and COREY R. B. (1953) *Proc. Nat. Acad. Sci.* **39**, 84; (1953) *Nature, Lond.* **71**, 346.

PEACOCKE A. R. and SCHACHMAN H. K. (1954) *Biochim. Biophys. Acta* **15**, 198.

PEACOCKE A. R. (1954) *Proc. Int. Symp. Macromol. Chem., Milan-Turin.*

PEACOCKE A. R. (1955) *Ric. Sci.* **25**.

PEACOCKE A. R. and SKERRATT J. N. H. (1956) *Trans. Faraday Soc.* **52**, 261.

PETERLIN A. and SIGNER R. (1953) *Helv. Chim. Acta* **36**, 1575.

PETERLIN A. (1953a) *Makromol. Chem.* **9**, 244; (1953) *J. Polymer. Sci.* **10**, 425.

PETERLIN A. (1953b) *Nature, Lond.* **171**, 259.

PETERLIN A. (1954) *J. Polymer Sci.* **12**, 45.

POUYET J., SCHEEBLING G., and SCHWANDER H. (1950) *J. Chim. Phys.* **47**, 716.

POUYET J. (1952) *C. R. Acad. Sci., Paris.* **234**, 152.

POUYET J., HERMANS J., and VENDRELY R. (1957) *Trans. Faraday Soc.* **53**, 247.

REICHMANN M. E., BUNCE B. H., and DOTY P. (1953) *J. Polymer Sci.* **10**, 109.

REICHMANN M. E., RICE S. A., THOMAS C. A., and DOTY P. (1954) *J. Amer. Chem. Soc.* **76**, 3047.

REICHMANN M. E. (1956) *J. Phys. Chem.* **60**, 831.

ROWEN J. W. (1953) *Biochim. Biophys. Acta* **10**, 391.

ROWEN J. W., EDEN M., and KABLER H. (1953) *Biochim. Biophys. Acta* **10**, 89.

ROWEN J. W. and NORMAN A. (1954) *Arch. Biochem. Biophys.* **51**, 524.

SADRON C. (1955) *Proc. Third Int. Congress Biochem., Brussels*, p. 210.

SCHOLES G. and WEISS J. (1950) *Nature, Lond.* **166**, 140.

SCHOLES G. and WEISS J. (1952) *Exp. Cell Res. Suppl.* **2**, 219.

SCHOLES G., WEISS J., and WHEELER C. M. (1956) *Nature, Lond.* **178**, 157.

SCHUMAKER V. N. and SCHACHMAN H. K. (1957) *Biochim. Biophys. Acta* **23**, 628.

SCHUSTER V. H., SCHRAMM G., and ZWILLIG W. (1956) *Z. fur Naturf.* **116**, 339.

SCOTT J. F. (1948) *Biochim. Biophys. Acta* **2**, 1.

SETTON R. and DOYLE B. (1954) *Biochim. Biophys. Acta* **15**, 117.

SEVAG M. G., LACHMAN D. B., and SMOLENS J. (1938) *J. Biol. Chem.* **124**, 425.

SHACK J., JENKINS R. J., and THOMPSETT J. M. (1952) *J. Biol. Chem.* **198**, 85.

SHACK J., JENKINS R. J., and THOMPSETT J. M. (1953) *J. Biol. Chem.* **203**, 373

SHOOTER K. V. and BUTLER J. A. V. (1955) *Nature, Lond.* **175**, 500.

SHOOTER K. V. and BUTLER J. A. V. (1956a) *Trans. Faraday Soc.* **52**, 734.

SHOOTER K. V., PAIN R. H., and BUTLER J. A. V. (1956) *Biochim. Biophys. Acta* **20**, 497.

SHOOTER K. V. and BUTLER J. A. V. (1956b) *Nature, Lond.* **177**, 1033.

SHOOTER K. V. and BUTLER J. A. V. (1957) *J. Polymer. Sci.*, in press.

SHOOTER K. V. (1957) *Trans. Faraday Soc.* **53**, 247.

SMITH D. B. and SHEFFER H. (1950) *Amer. J. Res.* **28**, 96.

STACEY K. A. (1956) *Light Scattering in Physical Chemistry.* London. Butterworths.

THOMAS R. (1951) *Experientia* **7**, 261.

THOMAS R. (1953) *Bull. Soc. Chim. Biol.* **35**, 609.

THOMAS R. (1954) *Biochim. Biophys. Acta* **14**, 231.

THOMAS C. A. (1956) *J. Amer. Chem. Soc.* **78**, 1862.

THOMAS C. A. and DOTY P. (1956) *J. Amer. Chem. Soc.* **78**, 1854.

WATSON J. D. and CRICK F. H. C. (1953) *Nature, Lond.* **171**, 737; (1954) *Proc. Roy. Soc.* **A223**, 80.

THE PHYSICAL CHEMISTRY OF DEOXYRIBOSENUCLEIC ACID

FRANKLIN R. E. and GOSLING R. G. (1953) *Acta Cryst.* 6, 678; (1955), 8, 151.
FURBERO S. (1952) *Acta Chem. Scand.* 6, 634.

GEIDUSCHEK E. P. (1951) Ph.D. Thesis. Harvard University.
GEIDUSCHEK E. P. (1956) *J. Amer. Chem. Soc.* 78, 879.
GOLDSTEIN G. and STERN K. G. (1950) *J. Polymer Sci.* 5, 687.
GOODALL S. H., RUPERT C. S., and HERRIOT R. M. (1957) *Trans. Faraday Soc.* 53, 257.
GULLAND J. M., JORDAN D. O., and THRELFALL C. J. (1947a) *J. Chem. Soc.* 1129.
GULLAND J. M., JORDAN D. O., and TAYLOR H. F. W. (1947b) *J. Chem. Soc.* 1131.

HORN P., LERAY J., POUYET J., and SADRON C. (1952) *J. Polymer Sci.* 9, 531.
HURLEN E., LALAND S. G., COX R. A., and PEACOCKE A. R.; *Acta Chem. Scand.* (1956) *Acta Chem. Scand.* 10, 793.

IRVIN J. L. and IRVIN E. M. (1954) *J. Biol. Chem.* 206, 39.

JAMES D. W. F. (1954) Ph.D. Thesis. London University.
JORDAN D. O. (1951) *Progr. Biophys. Biophys. Chem.* 2, 51.
JORDAN D. O., MATHIESON A. R., and MATTY S. (1956) *J. Chem. Soc.* 158.

KAHLER H. and LLOYD B. J. (1953) *Biochim. Biophys. Acta* 10, 89.
KATCHALSKY A., SHAVIT N., and EISENBERG H. (1954) *J. Polymer Sci.* 13, 69.
KAY E. R. M., SIMMONS N. S., and DOUNCE A. L. (1952) *J. Amer. Chem. Soc.* 74, 1724.
KOENIG V. L. and PERRINGS J. D. (1953) *Arch. Biochem. Biophys.* 44, 443.
KUNITZ M. (1950) *J. Gen. Physiol.* 33, 349.
KURNICK N. B. (1954a) *J. Amer. Chem. Soc.* 76, 417; (1954b) 76, 4040.

LALAND S. G., LEE W. A., OVEREND W. G., and PEACOCKE A. R. (1954) *Biochim. Biophys. Acta* 14, 356.
LAWLEY P. D. (1956a) *Biochim. Biophys. Acta* 19, 160.
LAWLEY P. D. (1956b) *Biochim. Biophys. Acta* 22, 451.
LAWLEY P. D. (1956c) *Biochim. Biophys. Acta* 21, 481.
LEE W. A. and PEACOCKE A. R. (1953) *Research Suppl.* (6) 2, 155.
LUCY J. A. and BUTLER J. A. V. (1954) *Nature, Lond.* 174, 32.
LUCY J. A. and BUTLER J. A. V. (1955) *Biochim. Biophys. Acta* 16, 431.
LUCY J. A. (1956) Ph.D. Thesis London University

MANDELKERN L., KRIGBAUM W. R., SCHERAGA H. A., and FLORY P. J. (1952) *J. Chem. Phys.* 20, 1392
MASON S. F. (1954) *J. Chem. Soc.* 2071
MATHIESON A. R. and PORTER M. R. (1954) *Biochim. Biophys. Acta* 14, 288.
MATHIESON A. R. and McLAREN J. V. (1956) *J. Chem. Soc.* 303
MATHIESON A. R. and MATTY S. (1957) *J. Polymer Sci.* 23, 747
McCARTY M. and AVERY O. T. (1946) *J. Exp. Med.* 83, 97
MORTHLAND F. W., DE BRUYN P. P. H., and SMITH N. H. (1954) *Exp. Cell Res.* 7, 201

OSTER G. (1950) *Trans. Faraday Soc.* 46, 794
OTH J. and DESREUX V. (1954) *Proc. Symp. Macromol. Chem. Milan-Turin,* 447
OTH A. (1955) *Bull. Soc. Chim. Belg.* 64, 484

PAIN R. H. (1956) Ph.D. Thesis London University

REFERENCES

PAULING L. and COREY R. B. (1953) *Proc. Nat. Acad. Sci.* 39, 84; (1953) *Nature, Lond.* 71, 346.

PEACOCKE A. R. and SCHACHMAN H. K. (1954) *Biochim. Biophys. Acta* 15, 198.

PEACOCKE A. R. (1954) *Proc. Int. Symp. Macromol. Chem., Milan-Turin.*

PEACOCKE A. R. (1955) *Ric. Sci.* 25.

PEACOCKE A. R. and SKERRATT J. N. H. (1956) *Trans. Faraday Soc.* 52, 261.

PETERLIN A. and SIGNER R. (1953) *Helv. Chim. Acta* 36, 1575.

PETERLIN A. (1953a) *Makromol. Chem.* 9, 244; (1953) *J. Polymer. Sci.* 10, 425.

PETERLIN A. (1953b) *Nature, Lond.* 171, 259.

PETERLIN A. (1951) *J. Polymer Sci.* 12, 45.

POUYET J., SCHEIBLING G., and SCHWANDER H. (1950) *J. Chim. Phys.* 47, 716.

POUYET J. (1952) *C. R. Acad. Sci., Paris.* 234, 152.

POUYET J., HERMANS J., and VENDRELY R. (1957) *Trans. Faraday Soc.* 53, 247.

REICHMANN M. E., BUNCE B. H., and DOTY P. (1953) *J. Polymer Sci.* 10, 109.

REICHMANN M. E., RICE S. A., THOMAS C. A., and DOTY P. (1954) *J. Amer. Chem. Soc.* 76, 3047.

REICHMANN M. E. (1956) *J. Phys. Chem.* 60, 831.

ROWEN J. W. (1953) *Biochim. Biophys. Acta* 10, 391.

ROWEN J. W., EDEN M., and KAHLER H. (1953) *Biochim. Biophys. Acta* 10, 89.

ROWEN J. W. and NORMAN A. (1954) *Arch. Biochem. Biophys.* 51, 524.

SADRON C. (1955) *Proc. Third Int. Congress Biochem., Brussels,* p. 210.

SCHOLES G. and WEISS J. (1950) *Nature, Lond.* 166, 140.

SCHOLES G. and WEISS J. (1952) *Exp. Cell Res. Suppl.* 2, 219.

SCHOLES G., WEISS J., and WHEELER C. M. (1956) *Nature, Lond.* 178, 157.

SCHUMAKER V. N. and SCHACHMAN H. K. (1957) *Biochim. Biophys. Acta* 23, 628.

SCHUSTER V. H., SCHRAMM G., and ZWILLIG W. (1956) *Z. fur Naturf.* 116, 339.

SCOTT J. F. (1948) *Biochim. Biophys. Acta* 2, 1.

SETTON R. and DOYLE B. (1951) *Biochim. Biophys. Acta* 15, 117.

SEVAG M. G., LACHMAN D. B., and SMOLENS J. (1938) *J. Biol. Chem.* 124, 425.

SHACK J., JENKINS R. J., and THOMPSETT J. M. (1952) *J. Biol. Chem.* 198, 85.

SHACK J., JENKINS R. J., and THOMPSETT J. M. (1953) *J. Biol. Chem.* 203, 373.

SHOOTER K. V. and BUTLER J. A. V. (1955) *Nature, Lond.* 175, 500.

SHOOTER K. V. and BUTLER J. A. V. (1956a) *Trans. Faraday Soc.* 52, 734.

SHOOTER K. V., PAIN R. H., and BUTLER J. A. V. (1956) *Biochim. Biophys. Acta* 20, 497.

SHOOTER K. V. and BUTLER J. A. V. (1956b) *Nature, Lond.* 177, 1033.

SHOOTER K. V. and BUTLER J. A. V. (1957) *J. Polymer. Sci.,* in press.

SHOOTER K. V. (1957) *Trans. Faraday Soc.* 53, 247.

SMITH D. B. and SHEFFER H. (1950) *Amer. J. Res.* 28, 96.

STACEY K. A. (1956) *Light Scattering in Physical Chemistry.* London: Butterworths.

THOMAS R. (1951) *Experientia* 7, 261.

THOMAS R. (1953) *Bull. Soc. Chim. Biol.* 35, 609.

THOMAS R. (1954) *Biochim. Biophys. Acta* 14, 231.

THOMAS C. A. (1956) *J. Amer. Chem. Soc.* 78, 1862.

THOMAS C. A. and DOTY P. (1956) *J. Amer. Chem. Soc.* 78, 1854.

WATSON J. D. and CRICK F. H. C. (1953) *Nature, Lond.* 171, 737; (1954) *Proc. Roy. Soc.* A223, 80.

WILKINS M. F. H., STOKES A. R., and WILSON H. R. (1953a) *Nature, Lond.* 171, 738.

WILKINS M. F. H. (1953b), SEEDS W. E., STOKES A. R., and WILSON H. R. *Nature, Lond.* 172, 759.

WILLIAMS R. C. (1952) *Biochim. Biophys. Acta* 9, 237.

ZAMENHOF S., ALEXANDER H. E., and LEIDY G. (1953) *J. Exp. Med.* 98, 373.

ZAMENHOF S., GRIBOFF G., and MARULLO N. (1954a) *Biochim. Biophys. Acta* 13, 459.

ZAMENHOF S., LEIDY G., and REINER B. (1954b) *Proc. Amer. Ass. Cancer Res.* 1, 53.

ZAMENHOF S. (1950) *Progr. Biophys. Biophys. Chem.* 6, 85.

A COMPACT AND INEXPENSIVE TISELIUS ELECTROPHORESIS APPARATUS

by

L. O. BUTLER, D. W. F. JAMES AND J. D. TREHERNE

*Bacteriology Deb........ St C.....'. I. . . . . .,. : . . . .
T. . . . . . .
and Physic .· . . . . .*

There is a considerable need for a compact and inexpensive Tiselius electrophoresis instrument which does not sacrifice all the precision and sensitivity of the larger commercial models. Such an instrument should also be easy to manipulate and capable of giving results on small volumes in a comparatively short time. This paper describes the details of construction and performance of an instrument of this type which has been constructed in a laboratory workshop at low cost.

The 2.0 ml electrophoresis cell as used in the electrophoresis apparatus described by MOORE AND WHITE[1] (usually known as the Perkin-Elmer apparatus) was considered of suitable size, and since its design had already taken account of such problems as rapid heat transference and streaming, the cell was adopted as the starting point about which the instrument was to be designed. The Perkin-Elmer instrument has certain disadvantages which we have attempted to eliminate in the apparatus here described, but its advantages over the larger and more cumbersome instruments have been retained, *viz.*, the small thermostatic bath, simple cell assembly and smaller overall size (MOORE AND WHITE[1]). The question of overall length, however, must be a matter of compromise since the sensitivity is reduced as the optical path length is shortened. The Perkin-Elmer apparatus is compact because both the focal lengths of the schlieren lenses and the size of the water-bath are reduced. However, the sensitivity of the optical system is directly proportional to the focal length of the second schlieren lens. For this reason we have used a comparatively long focal length lens, as is usual in the larger instruments, with a consequent increase in total length compared to the Perkin-Elmer, but we have retained the small water-bath. The other main disadvantages of the Perkin-Elmer apparatus are the use of the framework and water-bath for mounting the optical components and the use of a plain slit as the schlieren diaphragm. These have been overcome firstly by mounting almost all the components on an optical bench bolted to an H-girder, thus ensuring that not only are the parts rigidly mounted without fear of distortion or disalignment from external stresses, but also each part is an independent unit and can be adjusted without causing disturbance to other parts. The only component that is mounted on the framework is the camera back. Secondly, a bar has been adopted as the schlieren diaphragm since ALBERTY, ANDERSON AND WILLIAMS[2] showed that with a knife-edge the area of the peaks is dependent on the exposure time used. This is due to the diffraction band moving into the light area as the exposure is increased. The bar is used in conjunction with the Philpot-Svensson cylindrical lens optical system (PHILPOT[3], SVENSSON[4, 5])

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in preference to the Longsworth scanning system (LONGSWORTH[4]) with its mechanical disadvantages.
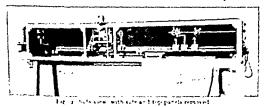
DESCRIPTION

The general view of the apparatus can be seen in Figs. 1,2 and 3. In Fig. 3, A and B are sections of 3 inch H-girder and C is the optical bench rigidly mounted on A. D

Fig. 1. End view. Letters refer to parts of the apparatus described in the text.

is the lamphouse, which, to prevent overheating, is mounted externally to the rest of the apparatus, the framework of which is shown by the dotted line. G is the slit which is at the focus of the first schlieren lens, H. Parallel light is therefore passed through

Fig. 2. Side view, with side and top panels removed.

the water bath J and then through the electrophoresis cell, and is brought to a focus by a second schlieren lens, K, onto the schlieren bar, L. M is a camera lens projecting

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an image of the cell on the photographic screen P, while N is the cylindrical lens focussing vertical lines in the plane of L onto the screen P. O is a photographic shutter. Figs. 1 and 2 also show the stirrer motor Q and the cable release R to the shutter. S is the power pack. The top panels and those of one side are removable.

The optical system uses a 125 watt mercury discharge lamp as the light source. The light is brought into a parallel beam by the convex lens E (Fig. 3) and then
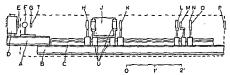

Fig. 3 Diagram of side view. Letters refer to parts of the apparatus described in the text.

focussed as a sharp line onto the slit G by a cylindrical perspex lens (F). The slit is adjustable, and was found to give good results when set at 0.005 inch. T is a filter allowing light of the wavelength of the mercury green line to pass. The two schlieren lenses H and K are 2½ inches in diameter and 25 inches in focal length, and are mounted on the optical bench. Parallel light is passed through the water-bath by means of four optical flats (U) set in the sides of the bath. The schlieren bar is 0.011 inch in diameter and is mounted on a cylinder capable of rotation and is graduated in degrees. The setting of the bar can be viewed through a telescope (V, Fig. 1) set in the lid of the instrument case. The angle of the bar, and hence the sensitivity of the instrument, is controlled by a knob situated by the viewing screen P. The camera lens is a convex lens of focal length 14 inches, and produces an image on the screen at a 1:1 magnification. The cylindrical lens N, focal length 4.5 inches, is mounted in a rotatable holder. The viewing screen is situated in a camera back, which is designed to take double-dark film holders. The instrument was focussed using methods described by LONGSWORTH[7].

The electrophoresis cell and cell-holder, buffer bottles and electrodes are as described by MOORE AND WHITE[1] using the open type system. The only modification we have made is that on the appropriate buffer bottle a cover is placed with a hole in it for the electrode wire to pass through, and also a tube having one end dipping into the buffer and the other end carrying a tap. This is connected by polythene tubing to an electrolytic compensator (JOHNSON AND SHOOTER[8]), the design of which is shown in Fig. 4. A is two platinum electrodes immersed in dilute sulphuric acid contained in tube B which itself fits into the outer bottle C by means of the ground glass joint D. Holes have been blown round the top of tube B just below the joint. Buffer is placed in bottle C which is then connected by the polythene tubing attached to the side arm E to that electrophoresis electrode vessel which is connected to the descending limb. A tap set in the polythene tubing allows buffer to be sucked up from both the bottles thus giving an unbroken liquid connection. The electrolysis of the diluted sulphuric acid is controlled from a switch and rheostat situated next to the viewing screen so that the movement of the boundary is easily seen and can be controlled at the same time. When the boundary has reached the required position the current is switched

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in preference to the Longsworth scanning system (LONGSWORTH[5]) with its mechanical disadvantages.

DESCRIPTION

The general view of the apparatus can be seen in Figs. 1,2 and 3. In Fig. 3, A and B are sections of 3 inch H-girder and C is the optical bench rigidly mounted on A. D

Fig 1 End view Letters refer to parts of the apparatus described in the text.

is the lamphouse, which, to prevent overheating, is mounted externally to the rest of the apparatus, the framework of which is shown by the dotted line. G is the slit which is at the focus of the first schlieren lens, H. Parallel light is therefore passed through
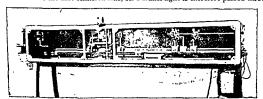

Fig 2 Side view, with side and top panels removed

the water-bath J and thus through the electrophoresis cell, and is brought to a focus by a second schlieren lens, K, onto the schlieren bar, L. M is a camera lens projecting

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bath with holes cut in the appropriate places to allow the various manipulations to be carried out with the lid in position.

The *power pack* used is a commercially available model giving a stabilised output of 0–200 V and 0–75 mA (Boulton-Paul Aircraft Ltd., Type E.P. 258 (A. 31)) Tappings are also available giving 6.3 V from which both the electrolytic boundary compensator and the lamp illuminating the scale on the schlieren bar are run.

RESULTS

The patterns obtained are illustrated by Figs. 6 and 7. Fig. 6 is of a normal human serum run in the NaCl/HCl/Na veronal buffer of MILLER AND GOLDER[1] at $\mu = 0.2$,
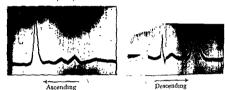

<div align="center">
←——— Ascending   Descending ———→
</div>

Fig 6   Electrophoretic patterns of serum, diluted 1 in 3, in Miller and Golder buffer,
pH = 8.5, $\mu = 0.2$ Time, 150 minutes

(a)

<div align="center">
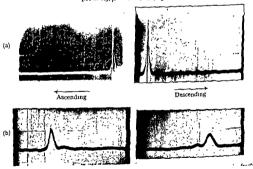←——— Ascending   Descending ———→
</div>

(b)

Fig 7

pH 8.5. The serum had been diluted 1 in 3. Fig. 7 is of a 1% (w/v) albumin solution run in the same buffer at $\mu = 0.2$ and pH 8.5. It can be seen that this solution also contains two globulin fractions as impurities, the smallest peak representing a concentration of about 0.02%. This illustrates the sensitivity of the apparatus. The

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off and the system is isolated by turning the tap above the buffer bottle. In this way the boundary can be shifted without disturbance and the operation requires no skill.

The water-bath is constructed of $\frac{3}{4}$ inch Perspex of dimensions $7\frac{1}{2}'' \times 7\frac{1}{2}'' \times 7''$,
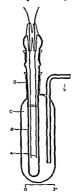

Fig 4 Diagram of the electrolytic compensator Letters refer to parts of the compensator described in the text

and is shown in Fig. 5. The two walls in the optical pathway each have a double-windowed cavity. The windows are of optical glass (A) and are held in position by rubber O-rings (B). The cavities are dried by silica gel containers (C) which are re-
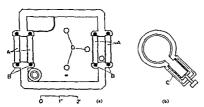

Fig 5 Diagram of the water-bath (a) Plan (b) End view of window showing silica gel container Letters refer to parts described in the text

placeable. Grooves (D) in the floor of the bath are arranged in such a way as to give kinematic alignment for the cell holder, which is then clamped firmly in position. A wire mesh in one corner forms a compartment for ice, and a perspex lid covers the

Reference p 183

РЕЗЮМЕ

Описываются конструктивные детали и рабочая характеристика улучшенного и дешевого электрофоретического аппарата Тизелиуса.

В аппарате применяются шаровые линзы сравнительно большого фокусного расстояния для достижения чувствительности, в то время как ·····························
обеспечив ·····················
с системо ····························

REFERENCES

1 D H Moore and J. W. White, *Rev. Sci. Instr.*, 29 (1948) 700.
2 R. A. Alberty, E. A. Anderson and J. W. Williams, *J. Phys. & Colloid Chem*, 52 (1948) 217.
3 J. St. L. Philpot, *Nature*, 141 (1938) 283.
4 H Svensson, *Kolloid Z.*, 87 (1939) 181.
5 H Svensson, *Kolloid Z.*, 90 (1940) 141.
6 L. G. Longsworth, *Ann. N.Y. Acad Sci*, 39 (1939) 187
7 L. G Longsworth, *Ind. Eng Chem. Anal. Ed*, 18 (1946) 219.
8 P. Johnson and E. M Shooter, *Science*, 109 (1949) 39.
9 G. L. Miller and R H Golder, *Arch. Biochem*, 29 (1950) 420

ADDENDUM

by

L. O. BUTLER AND A. M. STEWART

A sampling device for the removal of electrophoretic fractions

An instrument has been designed which will facilitate the removal of fractions from either limb of the electrophoresis cell. Its construction can be seen from Fig. 1.
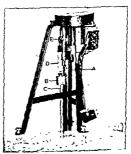

Fig 1 Electrophoretic sampler. Letters refer to parts of the apparatus described in the text.

A triangular framework is mounted on a slab of ⅜ inch Perspex which exactly fits the top of the water-bath of the electrophoresis apparatus. The framework supports a dove-tail slide (A) in which moves a saddle (B) driven by a 24 V.D.C. motor. The

TABLE I

AREAS OF THE PEAKS OBTAINED WITH THE ALBUMIN SOLUTION

1% (w/v) albumin solution in NaCl/HCl/Na veronal buffer, pH 8 5, $\mu = 0$ 2, run at 100 V, 17 5 mA

Time	Area: average of ascending and descending limbs (in arbitrary units)			
	Albumin	Globulin 1	Globulin 2	Albumin + Globulins
Initial	–	–	–	188
140 min	156	31	3	190

negatives are sufficiently sharp for enlargement for quantitative measurements, and the peaks and straightness of the base-line are satisfactory. Table I shows figures for the areas of the peaks obtained with the albumin solution initially and after 140 minutes at 100 V and 17.5 mA.

SUMMARY

The details of construction and performance of an improved, compact and in-expensive Tiselius electrophoresis apparatus are described.

The apparatus utilises schlieren lenses of relatively long focal length in order to retain sensitivity, but a small water-bath ensures compactness of the instrument. A bar as the schlieren diaphragm is used in conjunction with the Philpot-Svensson cylindrical lens optical systems.

RÉSUMÉ

Les auteurs décrivent les détails de construction et de performance d'un appareil d'électrophorèse de Tiselius amélioré, compact et bon marché.

L'appareil emploie des lentilles de schlieren de distance focale relativement longue afin de conserver la sensibilité, mais un petit bain-marie assure le caractère compact de l'appareil. Comme diaphragme de schlieren on emploie une barre en combinaison avec les systèmes optiques à lentilles cylindriques de Philpot-Svensson.

ZUSAMMENFASSUNG

Die Einzelheiten von Bau und Leistung eines verbesserten, kompakten und nicht teueren Tiselius-Elektrophorese-Apparates werden beschrieben. Der Apparat verwendet Schlierenlinsen von relativ grosser Brennweite um die Empfindlichkeit zu bewahren, aber ein kleines Wasserbad macht das Instrument kompakt. Ein Stab wird als Schlierendiaphragma verwendet, zusammen mit den Philpot-Svensson'schen optischen Systemen mit zylindrischen Linsen.

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LOCALIZATION OF DEOXYRIBONUCLEASE IN TISSUE SECTIONS

A NEW APPROACH TO THE HISTOCHEMISTRY OF ENZYMES

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Received September 3, 1956

METHODS currently used to localize enzymes in tissues consist in dipping tissue sections into a solution containing a suitable substrate and precipitating the products of reaction in a visible form. These products must adhere onto the sections with a minimum of diffusion from the sites of enzyme activity [4, 8].

In the present work, a different approach was investigated. The method is to place tissue sections in contact with a film of gelatine containing a substrate and, after exposure, to stain the film for the remaining substrate. The latter should be attacked in the regions of the film overlying the areas of tissue possessing enzyme activity and retained in those parts covering inactive areas. Staining of the exposed film and comparison with the corresponding tissue sections should thus reveal the sites of enzyme activity in the tissue. Films composed of deoxyribonucleic acid (DNA) dispersed in gelatine were used here in order to localize the deoxyribonuclease (DNAase) present in the tissue sections.

MATERIAL AND METHODS

The essential steps of the gelatine–substrate film method are illustrated in Fig. 1. Fig. 1a shows the materials used in their relative positions immediately before exposing the gelatine–DNA film to tissue sections. Fig. 1b represents a cross section of the same elements during exposure. Fig. 1c shows the tissue sections and the gelatine–DNA film after separation and staining. A negative image of the reactive portions of the sections is seen in the film.

Preparation of gelatine–DNA films.—A 1:1 mixture of 5 per cent gelatine (commercial sheet form) and 0.2 per cent DNA[2] is liquefied by heating in a water-bath. One

motor is controlled by a three-position switch situated by the viewing screen of the electrophoresis apparatus. The gearing on the motor gives a final movement of 1 cm in 7 seconds.

Through the centre of the saddle is drilled a tapered hole into which fits a syringe needle (C), which in turn is adapted to fit a luer syringe. The needle is 14.5 cm long and of 0.6 mm internal bore, except that the last centimetre is of 0.25 mm internal bore .The barrel of a 2 ml syringe (D) is fitted to the needle, the top being closed by a rubber bung carrying a small metal tap (E). To the tap is attached rubber tubing terminating in a syringe placed close to the viewing screen of the electrophoresis apparatus. This syringe serves to draw up the fraction. The framework is attached to the Perspex base by means of three knurled-topped screws, and alternative positions are available according to which limb is to be sampled.

With this apparatus, samples of any size may be taken from either limb. The position of the needle in the electrophoretic cell can be seen as a shadow across the screen, the edge of the shadow indicating the tip of the needle. Very small movement of the needle is possible so that a fraction may be sampled in a number of very small steps, thus reducing the possibility of mixing in the cell. The syringe drawing up the sample may also be finely controlled.

The sequence of operation, therefore, is as follows. After the electrophoresis is stopped, the position of the peaks are noted in terms of a scale attached to the viewing screen. The centre section of the electrophoresis cell is then removed out of alignment in order to isolate this section. The electrode is then removed from the side opposite to the limb to be sampled, and buffer solution added to fill both the top section of the cell and the electrode bottle, which are then sealed off by ground-glass plates. The buffer in the top section above the limb to be sampled is removed, and the sampler put into position. The centre section is then moved into alignment again, and the needle lowered till it just enters the centre section. Liquid is then drawn in to the needle, and when the required amount is drawn up, the tap on the sampler is closed and the needle withdrawn. The sample may then be pushed out into a suitable container. The process is then repeated until all the fractions required are removed. In this way, fractions are removed in a successive fashion down the cell. This obviates the need that LONGSWORTH[1] found for forcing air down the needle to remove unwanted liquid. In his arrangement, a glass capillary was lowered into the cell by a rack and pinion mechanism until the tip was level with the upper boundary of the desired layer, the meniscus in the capillary forced down to this level by injecting air from a syringe, and then the tip finally introduced into the layer to be sampled. With this procedure there must be quite a risk of causing mixing of the layers, a factor that the described apparatus and method has tried to eliminate as far as possible.

REFERENCE

1 L. G. LONGSWORTH, *J. Am. Chem. Soc.*, 67 (1945) 1109

the sections spread on its surface. The slide is then transferred onto a cool level surface (room temperature) and the gelatine–glycerol support is again allowed to form a firm gel (10–20 minutes). This procedure of allowing the support to liquefy and reset prevents the sections from adhering to the gelatine–DNA film when the slides are separated after exposure.

Exposure of gelatine–DNA films to tissue sections.—The slide carrying the sections is then placed against the dry gelatine–DNA film and the two are held in close contact by pressure. After standing at room temperature for a given period of time, they are separated again. The gelatine–DNA film is washed in two successive baths of distilled water, 5 minutes each, and allowed to dry. The slide supporting the tissue sections is placed in neutral 10 per cent formaldehyde, in a horizontal position, and left in the fixative overnight. It is then washed in two successive baths of distilled water, 5 minutes each, and left aside until the gelatine–DNA support has dried.

Staining of gelatine–DNA films and tissue sections with toluidine blue.—The dry gelatine–DNA film is dipped into a 0.2 per cent solution of toluidine blue for 10 minutes. The slide is then washed in distilled water to remove excess dye, the film is allowed to dry again and directly mounted with Canada balsam. Control films (2.5 pe᛫ᛘᛘ ᛘ᛫ᛘᛘᛘ᛫ ᛘᛘᛘᛘᛘᛘ

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medium which contains a higher amount of gelatine than the gelatine–DNA film. The slide is transferred directly from acetic alcohol to a 0.1 per cent solution of toluidine blue. After 1–2 minutes in the latter solution, the slide is washed in distilled water to remove excess dye, allowed to dry at room temperature and mounted by adding a drop of Canada balsam and covering with a coverslip.

RESULTS

Effect of DNAase solution on gelatine–DNA films.—Formaldehyde-fixed gelatine–DNA films treated with a solution of DNAase[1] at 37°C showed a progressive loss of DNA with time (Fig. 2). While a slight effect was observed after 2 hours, treatment for 8 hours resulted in an appreciable decrease in stainability, and a complete removal of the DNA was observed after 24 hours. The control solution had no effect on the films in the same conditions.

Effect of tissue sections on gelatine–DNA films.—When exposed to tissue sections, the gelatine–DNA films showed a loss of DNA in those regions in contact with sections. Fig. 3 illustrates some of the negative patterns left in a film after exposure to sections of the *small intestine* (Fig. 4) for 1 hour and staining with toluidine blue. The negative *images* corresponding very well to the sections are due to the removal of DNA which is attributed to the hydrolytic action of the DNAase contained in the tissue sections.

[1] The DNAase solution was prepared by adding 0.1 mg of crystalline DNAase (Worthington Chemical Lab.) per ml of McIlvaine's buffer at pH 6 5 containing magnesium sulfate at a final concentration of 0 005 *M*. The control solution contained the same reagents but no DNAase.

drop (ca. 0.05 ml) of the mixture is placed on a glass slide and rapidly spread over a surface of about 2.5 × 4 cm with the tip of the pipette. The slide is left on a level surface at room temperature until the gelatine–DNA gel has dried. The film is then fixed overnight in neutral 10 per cent formaldehyde to render the DNA insoluble in water. After fixation, the film is washed in three successive baths of distilled water, 15 minutes each, and allowed to dry.
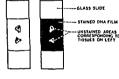

Fig. 1. Localization of DNAase in tissue sections by the gelatine–substrate film method. (a, top), materials used in their relative positions immediately before exposing the gelatine–DNA film to tissue sections. (b, center), cross section of the same elements during exposure. (c, bottom), tissue sections and gelatine–DNA film after separation and staining. A negative image of the reactive portion of the sections is seen in the film.

Preparation of tissues.—Male albino rats (ca. 200 g) are killed and the organs excised as rapidly as possible. The organs are placed in a beaker kept on ice, washed in distilled water and blotted on filter paper. A piece of each organ is cut out with a scalpel and placed immediately on a layer of ice covering a freezing stage dipped into cracked dry ice. When the tissues are frozen, the stage is adjusted on the freezing microtome kept in an insulated box at ca. −20°C and sufficient time is allowed for the tissues to adjust to this temperature.

Mounting of tissue sections on gelatine–glycerol.—When sections are ready to be cut, a mixture of gelatine and glycerol (ref. 1; final concentrations used: 7 and 40 per cent respectively) is liquefied by heating in a water-bath. About 0.3 ml of this mixture are placed on a glass slide and spread over a surface of approximately 2.5 × 4 cm with the tip of the pipette. The slide is left on a level surface at room temperature until gelation occurs (5–10 minutes).

Frozen sections are then cut at 15 μ according to the techniques of Linderstrøm-Lang, Holter and Mogensen (as described in [3]) and the sections are slid with a fine brush from the microtome knife onto the gelatine–glycerol-covered slide. The latter is introduced into the cold box only for the time necessary to transfer the sections in order to prevent freezing of the semi-solid support. The slide carrying the tissue sections is placed on a level surface at 37°C until the gelatine–glycerol liquefies and
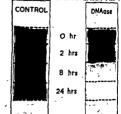
Fig. 2. Gelatine–DNA films treated with control or DNAase solutions for different periods of time and stained with toluidine blue.

Fig. 3 Gelatine–DNA film exposed to sections of the small intestine for 1 hour and stained with toluidine
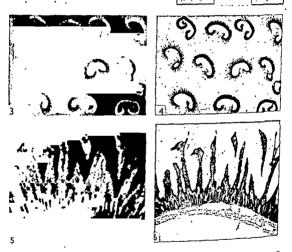
Fig. 5. Part of a negative image left in a gelatine–DNA film exposed to sections of the small intestine for one hour (Fig 3) seen at higher magnification (×40).

Fig 6. Corresponding tissue section at same magnification.

Examination of sections and corresponding autographs at higher magnification (using a yellow filter) reveals that the DNAase activity is shown mainly by the epithelial cell layer of both the villi and the crypts of Lieberkühn (Figs. 5 and 6). Weaker reactions are given by the lamina propria, the submucosa and the muscle layers but an intense reaction, assigned to a blood vessel, is also observed in the submucosa. The reaction associated with the villi epithelium is discontinuous along the epithelial cell layer and clearly extends beyond the surface into the lumen. The presence of DNAase in the lumen is particularly evident where the spaces between the villi are relatively narrow. These spaces appear filled with the enzyme. The areas of activity associated with the crypts of Lieberkuhn do not seem, on the other hand, to cover the entire diameter of the crypts. They rather correspond to the central part of the glands, i.e. the apical portion of the cells and the lumen. Presumably, the DNAase is contained in the excretory region of the goblet cells of both the crypts and the villi epithelia and is discharged into the lumen together with the mucus.

For studying the reaction given by the most active parts of a tissue, autographs of higher resolution can be obtained by reducing the time of exposure. Fig. 7 shows the negative image left in a film after contact with a section of the duodenum (Fig. 8) for 30 minutes, and Fig. 9 shows the results obtained by a 10 minute exposure to a section of the ileum (Fig. 10). These autographs clearly illustrate the presence of DNAase in the spaces between the villi. Mucus secretion can be observed in the same location in the corresponding sections. Moreover, focal reactions which may be assigned to individual goblet cells can be distinguished along the region of the villi epithelium, on each side of the active areas associated with the pits (Fig. 7, left part). These individual reactions are still more evident in some autographs of the crypts of Lieberkühn (Fig. 9, bottom left corner).

With the *pancreas*, a negative pattern corresponding to the acinar cells was obtained (Figs. 11 and 12). A multifocal reaction was sometimes observed in adjacent acini, suggesting that the DNAase is not evenly distributed in the acini but more concentrated in their centers, i.e in the secretory pole of the cells.

In the *thyroid*, DNAase activity was shown by the colloid present within the follicles, the intensity of the reaction varying from one follicle to another (Figs. 13 and 14) The follicular epithelium itself was relatively inactive. The interfollicular connective tissue showed a multifocal reaction which may be due to the small blood vessels present in this location.

DISCUSSION

This first attempt to localize enzymes in tissues by the use of gelatine-substrate films has proved successful and the new method seems to present many advantages. Firstly, fresh tissue sections can easily be used in this method, thus permitting to localize several enzymes which are inactivated or dissolved by the treatments necessary in the usual histochemical techniques [4]. Secondly, the demonstration of enzyme activity is here directly related to changes in the substrate instead of being associated with the formation of degradation products. This eliminates several problems encountered in the usual procedures such as suitable reactions for precipitating the end-products in a visible form, diffusion of these products from their sites of formation, adsorption on inactive parts of the tissue, etc. [2, 4, 5, 8]. In addition, the method offers the advantage of being applicable to biological fluids. Since films of substrate can be used with solutions as well as with tissue sections, they may also find applications in related fields: studies on enzymes specificity, comparisons between cellular and purified enzymes, etc.

A disadvantage of the method is its lower resolution, as compared with the *end-product precipitation methods which can reveal the intracellular dis-tribution* of enzymes. A localization at that level could sometimes be achieved by the present method in special cases where the cells present a par-ticular arrangement. The studies on the *small intestine*, for instance, have suggested that active DNAase is contained in the secretory region of the goblet cells and absent from their base. However, the method, in its present state at least, is not generally applicable to the cellular level. The resolution of the method depends upon several factors: the thickness of the film, the con-centration of the substrate in that film, the distance between the sections and the film, and the duration of the exposure. The composition of the semi-solid

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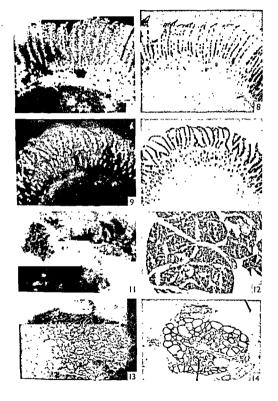
... to sections of the duodenum for 30

n for 10 minutes

creas for 1 hour

e thyroid for 5

satisfactory for this enzyme which was thus localized in the small intestine, pancreas and thyroid of the rat. The method appears applicable to the localization of other enzymes.

REFERENCES

1. BAKER, J. R., Cytological Technique. Methuen and Co., London, 1945.
2. DANIELLI, J. F., Cytochemistry. John Wiley and Sons, Inc., New York, 1953
3. ———— ———— ———— ———— ———— ———— ———— -ience Publ., Inc., New York, 1949
4.   t Chicago Press, Chicago, 1952
5.
6. KAY, E. R. M., SIMMONS, N. S., and DOUNCE, A. L., *J. Am. Chem. Soc.* **74**, 1724 (1952).
7. MARSHALL, J. M., *Exptl. Cell Research* **6**, 240 (1954).
8. PEARSE, A. G. E., Histochemistry. Churchill Ltd., London, 1953.

support for the sections is also important. It was observed that the addition of glycerol to the support greatly improved the precision of the autographic image, presumably by reducing the rate of enzyme diffusion. The results described above were obtained under the optimum conditions determined after series of assays. While the property of enzymes to diffuse through a thin film is actually the basis of the method, the optimum conditions will naturally be those which will minimize the geometrical affects of enzyme diffusion, a situation in many ways similar to that encountered in radio-autography.

The present approach, which has proved practical for localizing DNAase activity, is probably applicable to many different enzymes since suitable films of several substrates can likely be prepared. To visualize the unattacked substrate remaining in the film after exposure, any staining or other method can be used. This procedure does not need to be specific for the substrate, as long as the second component of the film, gelatine, does not stain by the same technique. Thus, the method seems to offer interesting advantages and should prove fruitful in various studies.

In comparing methods for localizing enzyme activities in tissues, no mention was made of the use of fluorescein-labelled antibodies [7]. In that method, the reaction localizes the enzymes as proteins, i.e. does not distinguish between the inactive and the active forms of enzymes, while the methods discussed above localize selectively the active fraction of these proteins. An interesting application of the antigen-antibody reaction might be its use in conjunction with methods based on the activity of enzymes to localize, by difference, the inactive or latent form of enzymes.

SUMMARY

A new approach to the localization of enzymes in tissues was investigated. The method is to place tissue sections in contact with a film of gelatine containing a substrate, to allow the tissue enzyme to act upon its substrate in the film and, after separating the sections and the film, to stain the latter for the remaining unattacked substrate. The latter is attacked in the regions of the film overlying the areas of tissue possessing enzyme activity and retained in those parts covering inactive areas. Comparing the negative pattern left in the film with the corresponding tissue sections reveals the sites of activity in the tissue.

Films made of deoxyribonucleic acid dispersed in gelatine were used in order to localize deoxyribonuclease in tissue sections. The method proved

an attempt to find changes during the very earliest stages of tumour growth. One striking change was found and is reported in this paper; it is shown however, to be characteristic of normal tissue growth as well as neoplastic.

METHODS AND MATERIALS

Antisera.—Two kinds of antisera were employed. For the first, 2 rabbits were injected with plasma from rats with large, actively growing, primary sarcomas induced with benzpyrene. The citrated plasma was emulsified in an equal volume of adjuvant consisting of 1·5 parts Arlacel A to 8·5 parts Bayol F. Four ml. of this emulsion was injected intramuscularly and this injection was repeated two weeks later. The animals were bled 11 days after the last injection. Ten months later a second antiserum was obtained from these rabbits after repeating the injection, this time subcutaneously. This last antiserum gave excellent discrimination between cancer and normal serum and was the one chiefly employed throughout the work. It was given the abbreviation A.K.P. (for anti-cancer plasma).

from a rat bearing a large transplanted sarcoma, were added 16 volumes of antiserum. After incubation for 2 hours at 37° C. and overnight at 6° C. the precipitate was centrifuged down and the supernatant used to immunize 2 rabbits in the manner described above except that inoculation was subcutaneous. A second antiserum was obtained from these rabbits some months later, after injecting an absorbed plasma obtained from a rat bearing a large hepatoma induced by feeding butter yellow. These antisera were given the abbreviation A.A.K.P. (for anti-absorbed cancer plasma.)

Animals.—All rats used were, unless otherwise stated, from a colony of albino animals originally derived from the Wistar strain. They had been bred by cousin matings. Males were used almost exclusively. They were bled from the heart under ether anaesthesia after overnight fasting. Rabbits were of various breeds obtained from dealers. They   vein.

Gel diffusion technique.—Ouchter   essentials.
One per cent New Zealand agar, clarified and dissolved in 0·8 per cent NaCl buffered at approximately pH 7·3, was employed. Merthiolate at a concentration of 1 part in 5000 was incorporated in both the agar gel and in the antigens and antisera. The petri dishes in which the reactions were being carried out were kept in air-tight glass jars at room temperature. Two main types of configuration of the wells in the agar gel were used, one with three wells (cf. Fig. 1), the antiserum being placed in the bottom well and the two plasmas which were being compared, in the top wells; the other configuration was a 5-well modification of the first, with two additional antigen wells added to form a square (cf. Fig. 2). Plasmas for use in the gel plates were routinely diluted with 14 parts of citrate-saline (4 per cent sodium citrate, 1 part : 0·85 per cent NaCl, 4 parts), and sera with 19 parts of 0·85 per cent NaCl. Antisera were used either at full strength or diluted with one part of 0·85 per cent NaCl. No refilling of the wells was done.
Photographs were taken by ultraviolet light on blue-sensitive film. The first photograph of each plate was usually taken at 2 to 5 days, after the start of the experiment, a second at 9 to 11 days, and a third at 2 to 3 months.

IMMUNOLOGICAL DEMONSTRATION OF A SUBSTANCE IN RAT BLOOD ASSOCIATED WITH TISSUE GROWTH

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IT was shown in an earlier paper (Darcy, 1955) that it is possible to distinguish the blood plasma of tumour-bearing rats from that of control rats by means of the gel-diffusion technique of Ouchterlony (1948). This technique is essentially the precipitin reaction carried out in a gel. A strong precipitating antiserum is prepared against the antigen, in this case rat plasma (a mixture of antigens), and the two reactants are allowed to diffuse towards one another through an agar gel. Precipitation of the antigen-antibody complexes occurs in the form of bands or lines, each of these representing one or more antigenic components of the original antigen mixture. Ouchterlony's method permits two such antigen mixtures to be compared in such a way that it is possible to tell at a glance whether there is a component in one which is absent from the other or whether a particular component is in substantially higher concentration in one than in the other.

The earlier study revealed several differences between the plasma of tumour-bearing rats and their controls, the most striking being : (1) The leading band of the normal plasma, i.e. the band that migrated in front of the others towards the antiserum, was considerably weaker in the cancer than in the normal blood. This band has since been found to be produced by serum albumin. (2) In the cancer plasma this albumin band was preceded by a group of faint lines (designated as "K" lines) which were not evident in the precipitin spectrum of the normal plasma. Their presence appears to be due partly to the receded position of the albumin band which thus "uncovers" them, and partly to higher concentration or higher speed of diffusion of the antigens they represent. (3) A line present in the cancer plasma spectrum which was absent from the normal. The substance which this line represents could be made to appear artificially in the plasma of normal rats by ' ' ' ' ' ' ' ' ' before bleeding. · · · ·
to be in consideraoly lower concentra · ' '
higher.

The above characteristics were observed in the plasma of rats with well-established tumours. They were not seen in animals bearing the Walker tumour until about 6 days after its transplantation. A preliminary study (Darcy, unpublished) has since been made of human blood and has revealed exactly similar changes in the serum of patients with well-established tumours. But when serum from patients with early breast cancers was examined none of these differences from the normal were observed. Furthermore sera from some non-neoplastic diseases showed changes very like those of advanced cancer sera. Accordingly, in the present investigation attention was returned to rat blood in

D. A. DARCY

increase was greater at 3 days than at 1 day, and later results showed that the concentration of the antigen in the serum of a rat bearing the tumour for 13 days was about twice as great as at 5 days. One of the 5-day plasmas was comparatively weak in the antigen, and autopsy notes stated that the tumour in this animal was much smaller and in poorer condition than in the other 5-day animals. This suggested that the graft in question may have been starting to regress as happens in a proportion of these Walker tumour grafts

To confirm the effect at 24 hours, 10 m·· ··· ···· ···· ···· period were bled. All pla··· · ··· · · ··· · ··· · · ··· · ··· · ···· · ··· ···· ···· ···· ····
(Fig. 3). Other differ·· · · · ·· · · · ·· ·· ··· ·· · · · · · · · · · ··· · · · · · · ·· · ·· · ·
antibody well) of the o· · ·· ·· · · ·· ··· · · · ·· · · · ·· ·· · · · · · · · · · · ·· · ·· · · ·
in the control, and (b) :·· first line of the spectrum (produced by albumin) was ·· ····· ···· i.e. nearer the
somewhat weaker in the cancer blood. · ·· also stronger in the cancer blood than

Yoshida sarcoma

Other Cancer Bloods

To see whether the same change occurred with othe·· · strains, the Yoshida sarcoma wa· ··· ··· strain in which it grows almost ·
stock rat. Animals were bled i·
of the tumour. Two normal ···· ···· rats of the ···· ···· ···· after transplantation plasmas. The gel diffusion test showed that the ·· · ··· ··· ···· ···· different from the control· ··· · ·· ··· · ·· · ·· ·· ·· ·· · · ·· the controls. The 2, 4, ·
the substance exactly as ···· ····ed with the Walker tumour (Fig. 4).

August tumour

This tumour which ··· ···· ···
·· ·gust strain rats as a mammary
· origin. It takes almost 2 weeks
·····compared with about ···· a measurable size and about 3 months to kill its
rats were implanted with the tumour using rather larger grafts than usual in
order to favour any possible role played by graft-necrosis in the antigen increase.
The rats were bled in pairs, at 1, 2, 4, 8, and 16 days. One ··· · · · · · · ·
batch provided control blood ·· Of ·· · · · · · · · · · · · · · · ·
day ones sho·· · · · · · · · ·
in the co
and bett
slightly ·
quantitat
16-day plasmas showed a strong increase of the antigen , by this time the tumours ···y could not be regarded as significant. Only the two
had reached a fair size, about 3 cm. × 2 cm. measured through the skin (Fig 5)
These results suggested that the increase of the antigen in the blood was in
some way associated with tumour size or tumour growth.

Benzpyrene-induced sarcomas

This experiment was performed to see whether the antigen increase occurred
with non-transplanted tumours, and if so, how early in the process of tumour

Discriminatory Power of the Antisera

The first object of the investigation was to see whether the pattern of precipitation shown by the blood serum from an animal with an early cancer differed in a characteristic way from that of normal rat serum. In practice, this amounted to a search for any asymmetry in the total precipitate pattern when plasma from rats bearing a 5-day-old Walker tumour implant was compared side by side with normal plasma. The first indication that any such difference existed was obtained when an antiserum against the absorbed cancer plasma (A.A.K.P.) was used. Fig. 1 shows the result. This antiserum gave a weak and simple spectrum of only 4 lines as opposed to the usual 20 or more. The absorption of the antigen used to prepare this antiserum had greatly reduced the number of its component antigens. The figure shows no lines present on the cancer side which are not also present on the normal side. The only apparent difference is that one of the 4 lines (the second, counting from the antiserum well) is broader on the cancer side and extends nearer to the antiserum well. This indicates that the substance responsible for this line is in higher concentration in the cancer blood than in the normal. This not very striking difference was subsequently confirmed using other 5-day Walker tumour plasmas and fresh normal plasmas.

When the antisera prepared against the untreated rat cancer plasma (A.K.P.) were tested, one of them, too, gave a discrimination between 5-day Walker tumour plasmas and control plasmas. The essential difference was again a line in the spectrum which was stronger on the cancer side than on the control side (Fig. 2). In this case the difference was more striking. Other, less striking, differences were also noted and will be indicated later. It was clear therefore, that both types of antisera showed that there was a substance present in normal rat blood which was in noticeably higher concentration in the cancer blood. The magnitude of this concentration difference was found to be considerable (see below). The question next arose whether the substance was the same with either antiserum. This could be tested by comparing the two antisera in a 3-well plate, with the antigen (Walker tumour plasma) in the bottom well. The result was a clear-cut "reaction of identity" or arc-formation between the two lines in question, indicating that the antigen that produced them was one and the same. For most subsequent experiments the second antiserum (A.K.P.) was used because it showed the above concentration difference more strikingly (due to its higher content of the corresponding antibody) and could discriminate smaller concentration differences, and because it showed concentration differences in other antigens.

Walker tumour 1 to 5 days old

The next step was to find how early after implantation of the Walker tumour the above change could be detected in the animal's blood. Ten rats were implanted subcutaneously with pieces of healthy Walker tumour by trocar. Twenty-four hours later 2 of them were bled from the heart, another pair were bled at 2 days, other pairs at 3, 4, and 5 days. The tumour grafts were examined at autopsy.

The plasmas of all these rats showed a strong increase in the antigen, as had the 5-day tumour plasmas. Results with the A.K.P. serum indicated that the

implants. One of the hosts bled at 4 days gave a clear-cut positive (Fig. 6);
it had been noted at autopsy to have a much larger graft than its companion rat.
One of the animals bled at 24 hours showed a concentration of the antigen which
was intermediate between the normal and the Walker tumour level. The rest
were negative.

Wound healing and tissue regeneration

The last results made it unlikely that the phenomenon of the antigen increase
was specific for tumour growth and suggested that it was associated with active
growth of any tissue. To test this hypothesis, regeneration experiments were set
up.

Ten rats had the left lobe of the liver removed and were bled in pairs at 24
hours, 2, 4, 8, and 16 days. This was designed to cover the main period of
regeneration. The mass of the liver is known to be restored by 14 days after
partial hepatectomy and the peak of mitotic activity occurs at between 2 to 8 days.
Mitotic activity has begun by 24 hours. The results were as follows: All the
hepatectomy plasmas except the 16-day ones were positive; furthermore, they
were strongly positive, except the 1-day specimens and one of the 8-day ones which
were relatively weak but nevertheless showed considerably stronger concentrations
of the antigen than the normal (Fig. 7). The 16-day plasmas were negative.

The experiment was repeated for kidney regeneration. Ten rats had one
kidney removed and were bled as above. The 1-day specimens were positive,
but weakly so. Of the 2-day specimens, one was weakly positive the other
strongly. Both the 4-day specimens were moderately strong positives. The 8-

EXPLANATION OF PLATES

Fi ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ Rat cancer plasma (K)
                                                    fls to meet the anti-
                                                    · is in the width of
                                                    bearing a 1-day-old

Walker tumour graft.

Fig. 2.—The cancer plasma (K) is from rats bearing 3-day-old Walker tumour grafts The
antiserum (A.K.P.) was prepared against cancer plasma. The two cancer plasmas give
a strong band of precipitate which is almost absent from the normal rat plasma (N).

Fi ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ ⁙ the normal rat plasma (N). The
                                                                              The

Fig. 4.—4-day Yoshida sarcomar at plasma (K) contrasted with normal rat plasma (N) to
show the stronger mid-line of the cancer plasma.

Fig. 5.—16-day August tumour plasma (K) showing a much stronger middle band than the
normal (N). The antiserum (A.K.P.) was here used in a dilution of 1·1.

Fi ⁙ and with a

Fig. 7.—Plasma from rats 8 days after partial hepatectomy (Hz) is contrasted with
rat plasma (N). The mid-band is much stronger than the normal in one of the hepatectomy
plasmas, but only slightly stronger in the other.

Fi ⁙ ⁙ Plasma from rats pregnant for 10 and 20 days is contrasted with the normal.
Fi ⁙

the K serum.

induction it would appear. Stock albino rats were implanted subcutaneously with small pellets of 3 : 4-benzpyrene which normally gives rise to a sarcoma in about 75 per cent of the rats. The first tumours appear at about $5\frac{1}{2}$ months after the implantation. Six of these rats were bled at $1\frac{1}{4}$ months when there was no trace of palpable tumour. All but one were negative in the gel test (i.e. showed no increase in the antigen). Five more rats were bled after $4\frac{1}{2}$ months. None had palpable tumours. All were negative in the gel test except one. This one was the only female present. Six more of these rats were bled at $5\frac{1}{2}$ months after implantation of the pellet. One of them had a small visible lump and another had a barely palpable lump around the pellet. The remaining rats showed no evidence of a tumour at this time. When the plasmas were tested, those from the 2 rats with palpable lumps, and plasmas from two of the other rats, showed definitely higher concentrations of the antigen than did the controls. But none showed very large increases.

Control Plasmas

The first experiments were carried out comparing the cancer plasma with normal rat plasma. The normal was invariably negative, i.e., never showed a concentration of the antigen in any way comparable to that in the blood of animals with the Walker tumour. Various other types of non-cancerous control plasmas were then taken to see if the phenomenon was cancer specific.
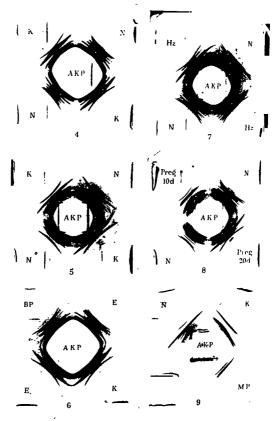
Bandaged and fed controls

The previous investigation had shown that when rats were bandaged around the thorax and abdomen, and were not fasted before bleeding, a new antigen appeared in their plasma. It was important, therefore, to see whether this procedure affected the antigen under consideration here. Ten rats were bandaged fairly tightly around the lower thorax and upper abdomen with gauze bandage, and over it plaster bandage about 2 inches wide. Another 10 were similarly bandaged but more in the abdominal region. Two rats from each batch were bled daily for 5 days. None were fasted before bleeding. Only one of the 20 plasmas showed a con · · .   . ·   ·   · higher than the con   · · · ·   ·   ;   · · · ·   · ·   ring the Walker tumour, fasting before bleeding did not affect the antigen increase.

Transplants of normal tissue

To obtain control animals comparable with the tumour-bearing ones, a series of grafting experiments were performed in which various normal tissues were grafted between rats of the albino stock. In the first experiment 10 rats were grafted subcutaneously by trocar with pieces of kidney from another rat. Two rats were bled out each day for 5 days thereafter. One of the 5-day specimens was " positive ", i.e. it showed a concentration of the antigen as strong as one of the weaker Walker-tumour plasmas. The rest were negative or else showed increases which could not be declared significant by the present method.

Another 10 rats were similarly grafted with embryonic tissue, a whole 8- or 9-day rat embryo being implanted subcutaneously in each host by trocar. All the plasmas were negative in the gel test. The experiment was then repeated using 19 to 20-day rat embryo liver in a volume comparable to the Walker tumour

Darcy.

Darcy.

experiment, a 13-day Walker tumour serum was examined and found to contain approximately 10 times as much of the substance as a normal control.

It could also be observed that the concentration of the antigen which was arbitrarily chosen as being " positive " (implying a significant difference from the normal) was certainly not less than 100 per cent greater than that in any of the normal plasmas studied.

Characterization of the Antigen

The first clue as to the nature of the substance was obtained when tests were done on a sample of rat serum albumin known to contain 4 per cent of α-globulin. This sample was prepared by means of ether fractionation and kindly supplied by Dr. Margaret Mackay. When tested in the Ouchterlony plates it gave several subsidiary bands of precipitate in addition to the main albumin one. It was found that one of the subsidiary antigens gave a reaction of identity with the antigen under study here. It was presumed therefore that the substance was probably an α-globulin. Confirmatory evidence was obtained by means of the technique of immuno-electrophoresis of Grabar and Williams (1953). Here it was found that the substance appeared to migrate with the α-globulins, although the possibility that it might be a fast-moving β-globulin could not be entirely ruled out.

A chemical approach was then made to the problem, and at the suggestion of Professor E. Boyland, rat cancer serum was precipitated with sulphosalicylic acid, and the supernatant, after suitable dialysis, was tested in the gel plates. It was found to give three bands, apart from traces of other antigens. One of the bands gave a reaction of identity with the antigen under study (Fig. 9). It can therefore be presumed to be a mucoprotein.

Site of Origin of the Substance

Preliminary studies have yielded no clue as to the site of origin of the substance. A saline extract made of non-necrotic, washed, and homogenized Walker tumour tissue contained no more of the substance than would be expected from the small amount of blood contamination still remaining. The same result was obtained with an extract of well-washed liver cells of a pregnant female rat.

DISCUSSION

The present experiments were begun largely in the hope of finding some characteristic early change in the blood of cancerous rats which might be of diagnostic value. No antigenic substance was found in the plasma of rats bearing early tumours which was not also present in normal rat plasma. However, one striking quantitative difference is reported, namely, an antigenic substance in normal blood which undergoes a large increase in concentration during tumour growth. Twenty-four hours after implantation of the Walker tumour the increase in concentration of this substance is approximately five-fold. When the slow-growing August tumour was employed no considerable increase took place until between 8 to 16 days after transplantation. During benzpyrene carcinogenesis the substance had already increased in the blood when the earliest tumours were present. Further experimentation showed that the phenomenon occurred in non-
⋯ ⋯ regenerating
⋯ ance was
also in distinctly higher concentration in the blood of 1-week-old ⋯ in in that
of 8-week-old rats.

day specimens were negative although one had a somewhat higher concentration of the antigen than the controls. The 16-day specimens were negative.

Finally, the experiment was repeated for skin regeneration. Ten rats were taken as before and a circular area of skin on their backs, 2·5 cm. in diameter, was excised in full thickness under sterile conditions. No sutures were applied but sterile *tulle gras*, gauze bandage, and finally the protective plaster bandage. The plasmas were tested as usual and gave the following results : 1-day plasmas, negative ; 2-day plasmas, one negative and one doubtful ; 4-day plasmas, positive but not strongly so ; 8-day plasmas, negative ; 16-day plasmas, one negative and one doubtful.

As a result of this finding sham operations were performed as if for hepatectomy. This procedure caused a definite but not large increase in the concentration of the antigen. The maximum concentration reached was not as great as that for the dermatectomy experiment. Nevertheless it suggested that a considerable part of the antigen increase in the nephrectomy experiment (where the increase was only moderate) was due merely to the laparotomy.

Pregnancy plasmas

As a further test for the hypothesis that the antigen increase was associated with actively-growing tissue, plasmas were tested from rats with pregnancies ranging from 10 to 20 days duration. All showed a very strong increase in the antigen—at least as strong as in the tumour blood. The weakest of the series was from the rat with the earliest pregnancy, namely 10 days (Fig. 8).

Immature versus mature rat blood

If the tissue growth hypothesis is true, it would be expected that the blood of young and rapidly-growing rats would have a higher concentration of the substance than that of adult rats. To test this, sera from rats, 1, 2, 4 and 8 weeks of age were compared in one gel plate. Four separate experiments were made. Each one showed that the blood of a 1-week-old rat had a distinctly higher concentration of the substance than that of an 8-week-old animal.

Quantitative Aspects

It was possible to obtain a rough estimate of the increase in concentration of the substance under study by two methods. The first was to take a normal plasma and find how high a concentration of it had to be employed in order to give a precipitate line identical with that in a cancer plasma used at the standard 1 : 14 dilution. The end-point was taken where the two lines were as nearly as possible equal in density, width, and distance from their own antigen wells. When a normal plasma was compared with a 1-day Walker tumour plasma in this way, it was found that the normal plasma had to be used in a 5 times higher concentration than the cancer plasma. The other method is one under development in this laboratory. In it, an extraneous " marker " line is introduced into the spectrum at a fixed point between the wells. The position of the line under study is noted in relation to the marker line and compared with its position in a series of standard dilutions. This method yielded the same result as the first, and indicates that the increase in concentration of the antigen under consideration is about five-fold at 24 hours after implantation of the Walker tumour. In another

tumour began to regress. This argues against the cell-division hypothesis proposed earlier, but it may be remarked (a) that besides the present antigen there are other mucoprotein components in rat serum which might be responsible for the high level and (b) it cannot be ruled out at once that there is less overall cell-division going on in the body after the tumour begins to regress—the activity of normal tissues, especially lymphoid and myeloid tissue, has to be taken into account.

Lockey, Anderson and Maclagan (1956) in a survey of the mucoprotein content of serum and urine report that high levels of these substances are frequently found in inflammation and in the collagen diseases as well as in cancer. Furthermore, surgical operations almost always produced a marked rise. In the light of the present results it is interesting to consider whether this rise in human serum mucoprotein might be due to one mucoprotein only, so that if this substance alone could be followed greater precision would result.

A recent report which is of great interest in the light of present findings is that of Nisselbaum and Bernfeld (1956). These authors isolated the α-globulin glycoproteins of the plasma of normal mice and tumour-bearing mice. The substances were studied chemically and physically and found to be distinct from one another. The protein preparation from the tumour-bearing animals behaved on electrophoresis like a nearly uniform substance over a wide range of pH, while that from normal mice was a mixture of several proteins. The authors conclude, " it appears likely that the α-globulin isolated from tumour-bearing mice is one of the regular constituents of normal mouse plasma α-globulin, the concentration of which increases during tumour growth ". The present findings in the rat are in harmony with this statement. On the other hand the earlier suggestion of Bernfeld and Homberger (1955) based on negative results obtained with normal and embryonic tissue grafts, that the increase of the plasma α-globulin is specifically associated with neoplastic growth, is opposed by the present results. It was found that if the growth of normal tissue was sufficiently vigorous, then positive tests were obtained.

Finally, there are one or two questions concerning the gel diffusion results that require discussion. The first is about certain assumptions made in interpretation of the precipitin lines. The assumption was made throughout that since the antigen under study gave a single line it was therefore a single substance. This need not necessarily be so. But since a wide range of antigen dilutions failed to split the line into components, the singleness of the antigen must be assumed until there is good evidence to the contrary.

The other assumption was that if a line is found to be nearer the antiserum well than another line with which it gives a " reaction of identity ", that the antigen responsible is in higher concentration in the first case. This is usually a safe assumption. However, if the first antigen should be of lower molecular weight (e.g. because it was partly hydrolysed) and therefore diffused faster than the other, the position-difference might be obtained without any actual increase of antigen. The author has had exactly this experience with the fibrinogen line of rat plasma which had become infected. However, in this eventuality the faster-diffusing antigen would be expected to give a line of different density and appearance, if not a reaction of " partial identity ". Since there was no evidence for this, the more straightforward interpretation was assumed here.

There were other antigenic changes noted in early tumour blood and it is hoped to present a fuller study of these at a later date.

It seems very likely therefore, that the increase in this substance is associated with active tissue growth rather than with a phenomenon such as tissue necrosis. For the necrosis hypothesis does not explain the negative results obtained with the majority of normal tissue grafts which certainly become necrotic. Nor could necrosis account for the positive results with pregnancy, regeneration, and 1-week-old rat plasma. On the other hand the increase of the substance appears to be related to the growth rate of the various tumours and to the extent and duration of the growth of normal tissue. The results suggest that the fundamental correlation might be with the overall mitotic rate of the body. The number of mitoses in a Walker tumour at 24 hours after transplantation could well be as great as in a much larger and later August tumour, or in a regenerating liver lobe. The failure to obtain a significant increase of the substance with most normal tissue grafts could be explained by their relatively low growth rates and the small amount of tissue involved. The level of the substance in normal blood could be accounted for by the mitotic activity needed for normal growth, maintenance, and repair. In short, the hypothesis that the concentration of the substance in the blood is directly associated with the total mitotic activity of the body seems worth considering.

It is more probable, however, that the fundamental correlation is with a secondary effect of tissue growth. Since the substance is apparently a muco-protein, it may be merely an expression of connective tissue activity. Connective tissue activity cannot be ruled out in any of the experimental situations described. In fact it is difficult to rule it out for any tissue growth *in vivo*. It is worth observing, however, that the high level of the substance in the blood during liver regeneration appears to be out of proportion to the visible fibroblast activity. The actual laparotomy can only account for a minor part of the increase.

The fact that the substance appears to be an α-globulin and a mucoprotein raises the question of its identity or otherwise with similar substances reported by other authors. Numerous reports are to be found in the literature of increases of α-globulins and mucoproteins in cancer and other conditions. Butler (1951) w~~~~~~ ~~ ~~ ~ ~ ~ ~ ~ ~~~~~~ ~ ~~~~~ ~ ~~~~~
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appeared to be responsible for the polarographic behaviour (Winzler and Šmyth, 1948), and this protein appeared to migrate with the α-globulins (Boyland, Butler and Conway, 1951). It appears likely therefore that the substance dealt with in the present paper may be the analogous substance in the rat to that dealt with in man by the polarographic method. However, since sulphosalicylic acid deproteinization of rat serum left at least three antigens, only one of which undergoes an increase during tissue growth and is the subject of the present paper, it seems that the gel diffusion method is more highly selective and could give more accurate results.

Shetlar (1952) states that the polysaccharide content of the albumin fraction of serum appears to be elevated in all cases in which tissue proliferation occurs but that the ~~~~~~~~~~~~~ ~~~~~~~ ~~ ~~~ ~-globulin fraction increases in fever. Th
 ·: · · ·:   ·   · · .· · , itudying the serum mucoprotein of rats bearing on ascites tumour found that it reached its maximum after the

FURTHER EVIDENCE FOR DIFFERENTIAL EFFECTS OF MUTAGENS IN *DROSOPHILA MELANOGASTER*

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1. INTRODUCTION

The early work on mutagenesis, mainly through the agency of radiation, seemed to indicate that the mutation process was random and indeterminate. This view is widely held by many geneticists, and its history has been recounted recently by Muller (1952, 1955). The analysis of mutagenesis in micro-organisms, however, did not support the principle of random mutability. The work of Demerec (1955) on *Escherichia coli* has revealed that the rate of reversion of several nutritional deficiency loci varied markedly under similar treatments with different mutagens. There was conclusive evidence also, that this differential response was a function of the genes themselves, rather than the cell environment, since there were no particular strains which were conducive to high, or low, mutation rates for all loci. The results with *Neurospora* were closely parallel to those in bacteria. Here again the rate of back-mutations of different loci, or even different alleles of the same locus, depended on the mutagen. This has been demonstrated for different radiations, such as ultra violet and X-rays (Giles, 1951), as well as for different chemical mutagens (Kölmark & Westergaard, 1953). That the differential response in *Neurospora* was a function of the locus has been shown by Kölmark (1953) on a double-mutant strain (adenineless and inositolless). Diepoxybutane proved to be markedly more effective on the adenine locus.

The evidence for selective mutagenicity in higher plants and animals has been very scanty, practically non-existent. In fact there were only the reports of the Gustafsson group (Gustafsson & Mackey, 1948; Gustafsson & Nybom, 1949); that the yield of mutations in cereals, especially barley, is dependent on the mutagen and the method of treatment. Quite recently, however, the work on the comparative mutagenic effects of the alkylating compounds and X-radiation (Fahmy & Fahmy, 1956a) has yielded the first decisive and comprehensive evidence for the differential action of various mutagens in *Drosophila*. This phenomenon does not only manifest itself in the different relative frequencies of the various types of mutations induced, but is sometimes of a much finer nature, being a specificity for certain gene loci. It has been shown, for example, that the 'N-mustard' derivative of phenylalanine(*p-N-di*(chloroethyl)aminophenylalanine) is most effective in the induction of 'visible' (morphologically detectable) mutations, and that most of these visibles are 'new', in the sense that they have not been recorded in the literature as induced by other mutagens.

The clearest differences in mutagenic effects, however, seemed to occur between chemical agents and radiation. In a recent paper (Fahmy & Fahmy, 1956a) we have outlined these differences on the basis of our results with the alkylating agents as com-

SUMMARY

An attempt has been made to discriminate the blood plasma of rats bearing very early tumours from that of control rats. The method used was the analysis of plasma antigens by means of the gel-diffusion technique of Ouchterlony (1948).

No antigenic substance was found in the plasma of rats bearing early tumours which was not also present in the control plasma. However, one striking quantitative difference is reported ; namely an antigenic substance in normal blood which undergoes a large increase in concentration during tumour growth. Twenty-four hours after implantation of the Walker tumour this increase is approximately five-fold. When the slow-growing August tumour was employed no substantial increase was observed until after 8 to 16 days following transplantation. During benzpyrene carcinogenesis the substance had already increased in the blood when the earliest tumours were present.

Further experiments showed that the phenomenon occurred in non-cancerous rats which possessed much actively-growing tissue, e.g. in animals regenerating liver, kidney or skin, and also in pregnant females. The substance was found in distinctly higher concentration in the blood of 1-week-old rats than in that of 8-week-old rats.

The substance appears to be a mucoprotein which migrates electrophoretically with the α-globulins. Its relation to similar substances reported on the literature is discussed.

The working hypothesis is suggested that the substance is directly associated with cell-division.

REFERENCES

BERNFELD, P. AND HOMBURGER, F.—(1955) Cancer Res., 15, 359.
BOYLAND, E., BUTLER, L. O. AND CONWAY, B. E.—(1951) Brit. J. Cancer, 5, 235.
BRDIČKA, R.—(1939) Nature, 139, 330.
BUTLER, L. O.—(1951) Brit. J. Cancer, 5, 225.
DARCY, D. A.—(1955) Nature, 176, 643.
GRABAR, P. AND WILLIAMS, J. A.—(1953) Biochim. biophys. Acta, 10, 193.
HOKKANEN, E., PYÖRÄLÄ, K. AND TAIPALE, E.—(1956) Acta path. microbiol. scand., 39, 15.
LOCKEY, E., ANDERSON, N. J. AND MACLAGAN, N. F.—(1956) Brit. J. Cancer, 10, 209.
NISSELBAUM, J. S. AND BERNFELD, P.—(1956) J. Amer. chem. Soc., 78, 687.
OUCHTERLONY, Ö.—(1948) Ark. Kemi. Min Geol., 26B, 1.
SHETLAR, M. R.—(1952) Texas Rep. Biol. Med., 10, 228.
WINZLER, R. J. AND SMYTH, I. M.—(1948) J. clin. Invest., 27, 617.

X-chromosome has been compared with that of the same mutations induced chemically in our own laboratory, as well as with Spencer & Stern's (1948) data (Table 1). Because of the smallness of our sample of X-ray lethals, the data had to be pooled very drastically, and it was thought that the most objective way would be a grouping between the common marker used in linkage experiments for each set of comparisons. The marker genes in our experiments with both the chemically and radiation-induced lethals were: acute (*sc*), cut (*ct*), vermilion (*v*), forked (*f*) and carnation (*car*). These are the same as those of Spencer & Stern (1948), except that the latter authors used cross-veinless (*cv*) instead of cut (*ct*). In comparisons between our data and those of Spencer & Stern (1948), we pooled the lethals between the common markers used in both sets of experiments, viz. *sc–v–f–car–centro-mere*. Needless to say that with such a grouping the relative frequency in the successive segments would be utterly objective and trustworthy, and completely independent of errors of placing within segments.

Table 1. *The distribution of the sex-linked recessive lethals induced by the alkylating compounds and X-radiation between the localization markers*

	sc	ct	v	f	car	centromere	
Mutagens and (authors)	0–19·9	20 0–32·9	33 0–56 6	56 7–62 4	62 5+		Total
Alkylating compounds (Fahmy & Fahmy, 1956a)	172	92	182	42	79		567
X-radiation (Fahmy & Fahmy)	24	16	21	6	3		70
X-radiation (Spencer & Stern, 1948)		116	80	21	12		229
Alkylating compounds (regrouped)		264	182	42	79		567
Pooled X-radiation data (Fahmy & Fahmy; Spencer & Stern, 1948)		156	101	27	15		299

Table 2. *The significance of the variation in distribution (expressed in χ^2) of the sex-linked recessive lethals induced by the alkylating compounds and X-radiation between the localization markers (based on data in Table 1)*

Genetical map of the X-chromosome

	sc	ct	v	f	car	centromere		
Comparison (and authors)	0–19 9	20 0–32 9	33 0–56 6	56 7–62 4	62·5+		Total χ^2	p
Alkylating compounds and X-radia-	4 563	1 945	0·126	0 121	5 170		11 925	0·018
		0 804	0 584	0 023	0 102		1 613	0 660
		1·096	0 594	0 695	12 16		14 545	0·004
Alkylating compounds and pooled X-radiation data		2 470	0 251	0 703	16·10		19 524	<0 001

It is convincingly clear from Table 2 that the distribution of the chemically and radiation-induced sex-linked recessive lethals is significantly different whichever data are used for the comparison. The greatest differences, on the basis of our own data, occur at the free and centromere ends of the chromosome. At the proximal end of the X-chromosome, near the centromere, the chemical agents induce more mutations than X-radiation, whereas at the distal free end the reverse is true, radiation being more effective.

pared to the radiation mutagenesis results available in the literature. The most obvious differences manifested themselves in the following respects:

(1) The frequency, properties, and distribution of the chromosome breaks.

(2) The mechanism of induction, and frequency of small deficiencies (involving up to 10 bands of the salivary gland X-chromosome).

(3) The ratio of 'visible' (morphologically detectable) to lethal recessive mutations induced in the same sample of treated chromosomes.

(4) The distribution of the genetically placed recessive lethals along the X-chromosome.

(5) The phenotypic expression and genetic position of the visibles induced.

In a note published in this journal, Stern (1957) has inquired into the degree of reliability of the last two of our criteria for difference between mutagens, viz. those based on genetical localization of lethals, and the detection of visibles. It would be of value, therefore, to examine whether any of the pitfalls he pointed out, could have affected our conclusions.

2. DISTRIBUTION OF RECESSIVE LETHALS

Stern (1957) has elaborated the well-known and accepted fact that heterogeneities of recombination do occur in linkage tests. It is equally known, however, that such heterogeneities can be reduced by adequate selection of gene markers, by the scoring of a reasonably high number of individuals, and by the use of optimal cultural conditions during experimentation. That is why, when we wanted to compare the distribution of the chemically induced lethals with the radiation mutations, we chose the Spencer & Stern (1948) linkage pattern, since their experimental procedure and criteria of accuracy were roughly the same as in our experiments. In both cases a five-point marker chromosome was used and a minimum of 200 males were scored for each lethal placing. Furthermore, like ourselves, these authors corrected the placings of their mutants in relation to the markers by reference to the standard map distances.

Stern (1957) objected to the comparison of the distribution of our chemically induced lethals with the X-ray mutations, on the grounds that the chromosome utilized in the placing of the radiation mutations gave recombination values between the utilized markers which varied more markedly from the standard map distances than was the case in our linkage experiments. This is undoubtedly true, but it is highly doubtful whether this heterogeneity could have resulted in any serious artifacts of distribution, particularly in the coarser groupings of our analysis. The average deviation from standard distances, even in the heterogeneous data of Spencer & Stern (1948), is 0.124 per unit recombination, with a maximum of 0.2 per unit, in the region cv (13.7)–v (33.0). It is hard to visualize how an error of that magnitude could be, (a) large enough or (b) unidirectional enough for a large number of the placed loci, to alter grossly the overall relative distribution of the chemical and radiation lethals among large segments (3 units in Table 6A and 12 units in Table 6B—Fahmy & Fahmy, 1956a).

It has always been realized however, that, the crucial evidence for the difference in the distribution of the sex-linked recessive lethals, under the effect of the radiation and chemical mutagens, must come from our own laboratory with work undertaken on both classes of mutagens with our own stock and with the comparison carried out on homogeneous data. This is now being undertaken and has already been partially accomplished. So far seventy X-ray sex-linked recessive lethals have been placed, and their distribution along the

did not differ significantly. Whether this situation holds true for the X-ray lethals as well, still remains to be seen. It would have been desirable to determine how different is the overall distribution of the corrected and uncorrected X-ray data of Spencer & Stern (1948) at different degrees of pooling. Unfortunately, however, the uncorrected data of these authors seem to be at present inaccessible.

3. 'VISIBLE' MUTATIONS

The detection of 'visible' mutations has long been recognized to depend to a large extent on the acuity of the observer. This large 'personal factor' in visible mutation work necessitates that the scoring should be undertaken by highly skilled workers with special aptitude for the recognition of morphological changes and with many years' experience with *Drosophila* material. In comparative mutagenesis results based on 'visible' detection, it is essential to restrict the scoring of the mutations induced by different agents to a few workers, preferably one. That is why, when the discovery of the differential yield of visibles by different mutagens passed the stage of impression and approached the stage of reality, we restricted all scorings of 'visible' mutations to one of us only (M.J.F.). For technical convenience all the mutation experiments in our laboratory are coded, and uncoding is not undertaken until the end of the experiments with each particular mutagen. Often experiments with various mutagens are interwoven and undertaken simultaneously, so as to make it impossible for the observer to predict which treatment the scored flies had received. All these precautions make the 'visible' data published from our laboratory as objective as is humanly possible, and they obviously meet the requirements stipulated in Stern's (1957) note.

In view of the above remarks it is considered that the strongest evidence for selective mutagenicity is demonstrated by the differential yield of 'visible' mutations by different agents. The ratio of visibles to lethals induced in the same sample of treated X-chromosomes varied considerably not only between radiation and chemical mutagens, but also between some of the different classes of the alkylating compounds themselves.

Most of these compounds induce 1 visible to 10 lethals. One exception is the 'N-mustard' derivative of phenylalanine, which yields 3 visibles to 10 lethals (Fahmy & Fahmy, 1956a). A new compound, viz. 2-chloroethyl methanesulphonate (Fahmy & Fahmy, 1956b), proved to be most effective in mutating morphogenesis loci, giving an overall ratio of visibles to lethals of 4:10. For the very young germ cells which are most susceptible to the action of this sulphonate the ratio of the two types of mutations reaches unity. A sample of X-ray mutations detected in the F_2 cultures of Muller-5 tests has also been analysed in our laboratory and by the same observer. In three experiments with various doses of X-rays the number of sex-linked visibles to lethals were 36:167, 10:84 and 15:95, giving an overall ratio of roughly 2 visibles to 10 lethals. This is the same ratio as that ascertained by Spencer & Stern (1948) in similar experiments with the same radiation.

Attention has already been drawn (Fahmy & Fahmy, 1956a) to the fact that the alkylating compounds seem to mutate 'new' morphogenesis loci which are different in phenotypic expression and genetic position from those induced by other agents. This selectivity for 'new' loci does not always go hand in hand with the overall effectiveness on the morphogenesis genes in general. Thus while 2-chloroethyl methanesulphonate proved to be the most effective agent in the induction of visibles, it is less selective on 'new' loci

It would be desirable to compare our own data for X-radiation and chemically induced lethals for various degrees of grouping along the X-chromosome. This is obviously not possible with the present small sample of X-ray lethals, but will undoubtedly be undertaken and published later. It was thought worth while, however, to pool our X-ray data with those of Spencer & Stern (1948), and compare the overall results with the data on the alkylating compounds for the same degree of grouping as was undertaken in our previous publication (Fahmy & Fahmy, 1956a), i.e. in 3-unit segments. The data were subjected to the Ψ test (Smith, 1952) as before, and both sets of analysis are presented in Table 3. It is of interest to note that the inclusion of our sample of lethals with that of Spencer & Stern (1948) has no pronounced effect on the degree of significance. This is further proof that the heterogeneity of the linkage data of the latter authors for the radiation mutations in comparison with our own data with the chemically induced lethals is perhaps not worth the emphasis given by Stern (1957).

Table 3. *The distribution of sex-linked recessive lethals along the genetic map of the X-chromosome under the effect of the alkylating compounds and X-radiation*

Genetical map of the X-chromosome (in 3 unit segments)

Mutagens (and authors)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Total	P
Alkylating compounds (Fahmy & Fahmy, 1956a)	100	10	16	17	17	9	9	23	33	20	10	21	18	17	27	22	24	29	32	27	27	26	26	7	567	0·008
X-radiation (Spencer & Stern, 1948)	31	12	3	1	10	4	13	15	10	11	6	9	16	7	10	6	11	11	19	9	4	7	4	0	229	0·003
Pooled X-radiation data (Fahmy & Fahmy and Spencer & Stern)	42	18	4	4	12	5	15	20	13	13	10	15	17	7	11	11	12	13	25	11	6	8	5	2	299	

Another point raised in Stern's (1957) note on linkage data concerns the effects of what he calls 'regional differentials in recombination' and how they might operate in producing artifacts of distribution in the process of correcting genetical placings to standard map distances. Of course, his arguments do not hold at all when comparisons of the linkage results are based on data pooled between the common markers used in placing experiments by different workers in the manner presented in the present paper. Yet, it is often desirable to make comparisons between the distributions of mutations placed by different workers without reference to the markers used. Most authors agree that in such cases it is satisfactory to give the position of the locus corrected to the standard map distance on the basis of the recombination values it gives with the marker genes on either side of it. As Stern (1957) points out, this procedure assumes that the variation in the recombination values occurring in the localization experiment is evenly distributed over the region between the marker genes. While this assumption is probably acceptable if the region between the markers is small, it could, if incorrect, be serious if the distance between the markers is large, as is the case with some segments in the lethal placings (v–f for example, covering a distance of 23·7 units). It must be pointed out, however, as Stern (1957) himself admits, that there is no experimental evidence whatever to help the assessment of the seriousness of regional differentials in recombination. We do know, however, that with the placings of the chemical lethals undertaken in our laboratory this phenomenon could not have caused serious disturbances in distribution. It was ascertained that the overall distribution of the corrected and uncorrected sex-linked lethals, even in the finest grouping tested (3 units),

only one has been affected intragenically by radiation and also eliminated within deletions; eight others have been eliminated in deletions, six at a low frequency, and two at a much higher frequency, but were not mutated intragenically. The remaining eight were stable to radiation in samples of the size utilized. That this size of sample is reasonably adequate is shown by the fact that it was sufficient for the induction of intragenic mutations, as well as deletions for the tested known loci, except *car* which was only eliminated.

Table 4. *Mutability at specific 'visible' loci under the effect of X-radiation*

'New' chemically mutated loci v. 1230	Genetic position	Mutation rate per locus per r × 10⁻⁸		Known loci (control)	Genetic position	Mutation rate per locus per r × 10⁻⁸	
		Deficiencies and deletions	Point mutations			Deficiencies and deletions	Point mutations
v. 1230	0 0	—		*scute*	0 0	1·74	3·49
v. 638	2 7		—				
v. 1276	4·1	1·24	—				
v. 1243	5·7	1·82	—				
v. 1998	6·3	1·27	—				
v. 1255	14·8	1·82	—				
v. 1287	23·4	1·52	—	*cut*	20 0	5 23	0·87
v. 1726	29 9	—	—				
v. 1892	40 5	1·17	—	*vermilion*	33 0	0·7	1·44
v. 1523	43·1	—	—	*wavy*	41·9	6 10	3 49
v. 2161	51·7	—	—	*garnet*	44 4	5·23	4 36
v. 2100	53 6	—	—				
v. 2212	59·1	—	—	*forked*	56·7	0 87	2 62
v. 1225	59·4	8·26	—				
v. 2073	61·6	1·05	8 42				
v. 1663	64·5	—	—	*carnation*	62 5	3·06	—
v. 1920	65 0	6 07	—				

Tested sample: 30,000–80,000 chromosomes per locus.
Dose range: 2500–4250 r.
v: symbol for 'visible' mutation.

The mutability of the same chemically induced and known visibles will be tested by the same technique under the effect of the phenylalanine mustard, and the full comparative results and their statistical analysis will be presented in due course. Nevertheless, it is perhaps worth mentioning that in a sample of 50,000 X-chromosomes treated with various alkylating compounds and scored in Muller-5 and attached-X experiments, all the 'new' visibles were detected at a minimum frequency of 2 and a maximum of 5. Most interesting is that *car*, which has not been mutated intragenically by X-rays in our sample (viz. 67,000 chromosomes for this locus), occurred 3 times in the chemical experiments (i.e. 3 in roughly 50,000 chromosomes). Also *sc* and *ct* which are mutated fairly frequently with radiation have never been encountered in the chemical experiments.

It can be safely concluded, even at this stage in our investigation, that the results of mutability at specific loci support the principle of differential gene response to mutagens. They also indicate that the detection of a large number of 'new' visibles under the effect of the alkylating compounds is a genuine reflexion of the higher response of some loci to these compounds and, therefore, perhaps not entirely due to a higher 'observational acuity'.

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than the 'N-mustard' derivative of phenylalanine. The percentage ratio of 'new' to known visibles under the effect of three alkylating compounds is as follows: p-N-di(chloroethyl)-aminophenylalanine, $80\cdot3\pm2\cdot0$:$19\cdot7\pm2\cdot0$; 2-chloroethyl methanesulphonate, $68\cdot5\pm3\cdot9$: $31\cdot5\pm3\cdot9$; 2:4:6-tri(ethyleneimino)-1:3:5-triazine, $59\cdot9\pm4\cdot2$:$40\cdot1\pm4\cdot2$.

Practically all the visibles referred to above as 'known' have been induced by radiation, especially X-rays. It must be emphasized, however, that the sample of X-radiation visibles recorded in the literature, or maintained in the private files of the radiation geneticists, is by no means a full list of all the mutations that can be induced by this agent. Actually as our own sample of X-ray mutations accumulates, it has become apparent that some of the 'new' visibles which are induced chemically can also be induced by radiation and must have been missed by the earlier workers. We discovered also some 'new' X-radiation visibles which were neither described by the earlier workers nor detected in our own work on chemical mutagenesis. These cases, however, are not sufficiently frequent to account for the fact that by the use of the alkylating mutagens we have nearly tripled the number of loci giving clear (rank 1 and 2) sex-linked recessive visibles (from 115 to more than 300) and that nearly two thirds of these have not been mutated by other mutagens including radiation, as far as can be ascertained.

4. MUTABILITY OF SPECIFIC 'VISIBLE' LOCI

To test how far the mutability of a locus is a function of the mutagen, we analysed the response of seventeen chemically induced visibles under the effect of radiation. The selected loci were spread along the whole length of the X-chromosome, were most easily recognizable, and each has occurred more than once in the chemical mutagenesis work. All mutants were fully viable and fertile in the homozygous condition. It was further possible to combine these mutants in sets of 2–4 loci per stock without impairing either the fertility or the viability. Care was taken to combine mutants which do not show any phenotypic overlap. For the sake of comparison we simultaneously tested the mutability of seven X-chromosome loci which are known to be affected (to various degrees) by radiation: viz. scute (sc), cut (ct), vermilion (v), wavy (wy), garnet (g), forked (f) and carnation (car).

Females, homozygous for the recessive visibles, or heterozygous for two sets of visibles one on each X, were mated to irradiated males carrying the normal allelomorphs, and the F_1 daughters were scored for the different genes tested. The number of chromosomes tested per locus is given by the total number of daughters when a homozygous mother is used, and only by half that number when the mothers are heterozygous. About 30,000–80,000 daughters receiving the marked and treated X-chromosomes were scored per locus. A daughter could show one of the characters tested for, if the paternal X has been affected so as to carry:

(a) a 'visible' allelomorphic to the marker, or (b) a 'visible' as above together with a lethal somewhere else on the chromosome, or (c) a deficiency or a deletion (in itself a lethal) covering the locus of the marker. Extensive genetic tests were undertaken to differentiate between the above three possibilities.

Table 4 summarizes the results. The left half of the table represents the mutability of the 'new' chemically induced visibles, whereas the right half represents the response of the known visibles for control purposes. Of the seventeen chemically mutated visibles

MUTAGENIC RESPONSE TO THE ALKYL-METHANESULPHONATES DURING SPERMATOGENESIS IN DROSOPHILA MELANOGASTER

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Introduction

T... diffe of t... is injected with a single dose of a chemical mutagen and then its germ line is fractionated by repeated matings to successive virgin females, the mutation-rate shows significant variations in the successive broods[1-2]. Since the sperm used in the successive matings must have been derived from progressively younger germ cells at the time of treatment, the fluctuations in the mutation-rate have been attributed to differential genetic response of the stages of spermatogenesis to the action of the mutagen. In this communication it is intended to analyse this pattern of genetic response to the action of different alkyl-methanesulphonates, and to reveal how far this analysis helps in the elucidation of the mechanism of mutagenesis.

Brood Technique and Cell-Stage Utilization

The testis of an adult *Drosophila* male presents an uninterrupted spectrum of differentiation from spermatogonia through spermatocytes and spermatids to mature sperm[4]. Any attempt at fractionating this continuous germ line to separate cell stages by repeated matings is bound to be very rough and approximate. Indeed, we have shown that even for the same mutagen and the same strain, considerable alterations in the brood-mutation pattern may occur in replicate experiments[3] in our laboratories. Nevertheless, it was possible to trace most of the factors responsible for the above variations, and modify our technique so as to eliminate or reduce the variables. Reasonable consistency has been achieved when great care was exercised in standardizing the experimental method, both in relation to the animals themselves and the mating procedure. We therefore developed a standard brood technique[3,4] in our laboratory and applied it for all the mutagens investigated. The males used were from the Oregon-K stock and were selected at an age of 30 ± 5 hr., and weighed 0.85 ± 0.05 mgm. The compound under test was dissolved in isotonic saline (0.4 per cent sodium chloride) and injected intra-abdominally around the testes. The concentration injected was as a rule well below the L.D 50-level. Each male received practically the same volume of solution, which was 0.25 ± 0.05 µl. The treated males were always mated 24 hr. after injection so as to allow the flies to regain their vigour and activity. The fractionation of the progeny into broods was ... ...-old females ... ...lay of treat-...ass cultures with a sex-ratio of unity. The cultures were kept at a constant temperature of $25 \pm 1°$ C. Under such conditions, it has been shown[5,3] that sperm ejaculated in the first four matings (4 broods: 12 days after treatment) has been derived from post-meiotic germ cells (sperm and spermatids), whereas sperm utilized in later matings (5th brood onwards: more than 12 days after treatment) has been derived from meiotic and premeiotic germ cells (spermatocytes and spermatogonia). The mutations which have been scored in the experiments to be reported here are the sex-linked recessive lethals and viables detected by the Muller-5 technique.
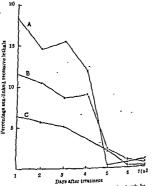

Fig. 1. Sex-linked recessive lethals in successive broods for ethyl methanesulphonate (curve A), and methyl methanesulphonate (curves B and C); based on data in Table 1

Brood-Mutation Pattern of the Alkyl Methanesulphonates

The mutation yield of the various stages of spermatogenesis under the effect of ethyl methanesulphonate (C.B. 1528 : $CH_3CH_2OSO_2CH_3$) and methyl methanesulphonate (C.B. 1540 : $CH_3OSO_2CH_3$) has been investigated by our standard brood technique, and the results are summarized in Table 1 and Fig 1. There is a convincing similarity in the brood-mutation pattern of the two sulphonates presented. The general tendency is that the mutation-rate decreases the later the brood. At the higher doses for both compounds (curves A and B, Fig. 1), and in spite of the non-significant fluctuations in the first four ... ...arper ...d the ... to the ...it the g. 1) the ...st linear fall in the mutation-rate is g... ... The decrease in from the first to the seventh broods [and, there the mutation-rate the later the brood fore, the younger the germ cell at the time of treat-ment) has already been ascertained also for the di-(methanesulphonoxy)alkanes[4]. It is, therefore, certain that the above general trend is characteristic of the various compounds of the alkyl methanesulphonate

5. SUMMARY

A sample of X-ray sex-linked recessive lethals has been localized by the same marker chromosome, and under identical experimental conditions, as prevailed in the placing of the same mutations induced by the alkylating compounds, so as to insure homogeneity of localization. The distribution of this sample of X-ray lethals along the X-chromosome varied significantly from that for the chemically induced mutations.

Differential production of visibles has been demonstrated by observations made by the same worker on coded experimental cultures. The ratio of visibles to lethals induced in the same sample of treated X-chromosomes, as well as the phenotype and genetic position of some of the visibles themselves, varied with the mutagen. Tests of mutability at specific chemically mutated visible loci under the effect of X-rays supported selective mutagenicity.

REFERENCES

Demerec, M. (1955). What is a gene? Twenty years later. *Amer. Nat.* 89, 5.

Fahmy, O. G. & Fahmy, M. J. (1956a). Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. V. Differential genetic response to the alkylating mutagens and X-radiation. *J. Genet.* 54, 146.

Fahmy, O. G. & Fahmy, M. J. (1956b). Mutagenicity of 2-chloroethyl methanesulphonate in *Drosophila melanogaster*. *Nature, Lond.*, 177, 996.

Giles, N. H. Jr. (1951). Studies on the mechanism of reversion in biochemical mutants of *Neurospora crassa*. *Cold Spr. Harb. Symp. Quant. Biol.* 16, 283.

Gustafsson, A. & Mackey, J. (1948). The genetical effects of mustard gas substances and neutrons. *Hereditas, Lund,* 34, 371.

Gustafsson, A. & Nybom, N. (1949). Colchicine X-rays and the mutation process. *Hereditas, Lund,* 35, 280.

Kølmark, G. (1953). Differential response to mutagens as studied by the *Neurospora* reversion mutation test. *Hereditas, Lund,* 39, 270.

Kølmark, G. & Westergaard, M. (1953). Further studies on chemically induced reversions at the adenine locus of *Neurospora*. *Hereditas, Lund,* 39, 209

Muller, H. J. (1952). Gene mutations caused by radiation. *Symposium on Radiobiology*. Nat. Acad. Sci. New York: J. Wiley and Son.

Muller, H. J. (1955). How radiation changes the genetic constitution. *Bull. Atom. Scient.* 11, 329.

Smith, C. A. B. (1952). A simplified heterogeneity test. *Ann. Eugen., Lond.,* 17, 35.

Spencer, W. P. & Stern, C. (1948). Experiments to test the validity of the linear r,-dose/mutation frequency in *Drosophila* at low dosage. *Genetics,* 33, 43.

Stern, C. (1957). A note on the detection of differential effects of mutagens. *J. Genet.* 55, 276.

residue, which is not used up in the alkylation of the cell constituents, almost certainly becomes inactivated (by hydrolysis) before the first sperm sample is drawn in the first minute (within three days after treatment). The mutation-rate detected in the successive broods would then represent the response of the genetic material of the various cell stages for roughly the same time of treatment. 2-Chloroethyl methanesulphonate, on the other hand, because of its lower ~~~~~~~~~ ~~ ~ ~ ~

such circumstances, the biological activity would be expected to rise as a function of the time-concentration product, and it would be feasible to arrive at a situation where the mutation-rate would increase with the time after treatment, which is roughly what was found experimentally for the first three broods. From the third to the fourth brood, however, there is a significant drop in the mutation-rate. A four-fold statistical test for this drop (illustrated in Fig. 2; for data see Table I, dose $1\cdot4 \times 10^{-2} M$, ref. 6) gave $\chi^2 = 4\cdot05$, $D.F. = 1$, $P = 0\cdot046$. This trough in the mutation curve at the fourth brood under the effect of the chloroethyl ester probably indicates the end of the influence of the intra-cellular 'residual' compound. If such an active compound was retained throughout the differentiation of the cells utilized in the fourth brood, they would be expected to yield a higher (or at least the same) mutation-rate as those recovered in the third brood, rather than a lower rate as observed. It seems, therefore, that no appreciable amount of active 'residual' sulphonate remains in the germ cells more than 9 days after treatment.

The most outstanding difference in the brood pattern of ethyl methanesulphonate as compared to the chloroethyl ester lies in the response of the early germ cells utilized later than the fourth brood. Whereas with the latter compound there is a sharp rise from the fourth to the fifth brood followed by the retention of a fairly high mutation-rate in later broods, the ethyl ester gives the exact opposite—a sharp drop from the fourth to the fifth brood and then a sustained low mutation-rate. The high mutation yield from the fifth brood under the effect of 2-chloroethyl methanesulphonate is probably not a function of time (that is, longer period of reaction between intra-cellular compound and the cell constituents), because of the presence of the trough in the mutation curve at the fourth brood. Two other explanations could, however, be postulated for the observed reversal of mutagenic efficiency on the early germ cells:

and differential
indirect action
secondary muta₋

two mechanisms are not necessarily exclusive, and a combination of them might actually occur.

If the hypothesis of direct action is to be adopted, then we shall have to assume a very high grade of specificity between the chemical structure of the mutagen and the physico-chemical state of the genetic material during differentiation; a specificity which is upset by a change in the alkyl group from ethyl to chloroethyl. It must be stated at once that there seems to be no evidence whatever for such a high grade of specificity. On the whole, alkylating agents of the same chemical series have roughly comparable brood-mutation patterns. Among the alkyl-methanesulphonates themselves, different compounds both mono- and di-functional (as regards the sulphonoxy groups) gave essentially the same cell stage-mutation pattern.

We are therefore ~ ~ ~ ~ ~
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'secondary mutagen' is produced in vivo which is most active on the early germ cells and which is not formed from other alkyl methanesulphonates, including the ethyl ester. Evidence for the setting in of mutagenesis through indirect action under the effect of the chloroethyl ester is not entirely lacking. Unlike the other sulphonates it gives a mutation-rate which increases with dose only for the later germ ~ ~ ~ ~ ~

administered and the mutation yield.

The time of production of the secondary mutagen is not difficult to deduce. The peak of maximal mutability attributed to this agent occurred in the sperm sample utilized in the fifth brood. It is in this sperm sample also that the first 'visible' clusters appear, indicating that this sperm must have been derived from the latest germ cells which were capable of multiplication, after mutating by the treatment. This means that the germ cells most responsive to the action of the secondary mutagen are the latest spermatogonia, just before they start metamorphosing to spermatocytes. Now, it is known that under our ~ ~ ~ ~ ~

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genetic effects of the secondary mutagen can be entirely accounted for on the basis of the time needed for the responsive cells to complete differentiation. In other words the secondary mutagen must have been formed, and produced its effect, almost immediately after the injection of the chloroethyl ester.

As to the chemical nature of the secondary mutagen, ~ ~ ~ ~ of ~~~~~~ and Warwick using ¹⁴C-ethyl

sulphonate under in vivo conditions is comparable with that of the ethyl ester, in which case the reaction product would not be S-ethyl cysteine, but its chloroethyl analogue.

Chloroethyl cysteine has been synthesized in the Chemistry Department of this Institute and will be analysed for mutagenicity, to determine whether it ~ ~ ~ ~ of the type yielded by

~ ~ ~ ~ ~—that is, ~ ~ ~ ~ ~ ~parable feature is that the phenylalanine mustard (particularly the L-derivative) yields a high ratio of visibles to lethals, which though not as high as for the chloroethyl ester, is nevertheless the nearest to it among the 'mustards' so far analysed[5,6].

To test the hypothesis of indirect action (through a metabolite) under the effect of 2-chloroethyl methanesulphonate we examined the brood mutation pattern for fluoroethyl methanesulphonate $(C.B. 1522,$ $FCH_2CH_2OSO_2CH_3)$. This latter compound has the

series, irrespective of whether the molecule is mono- or di-functional as regards the sulphonoxy groups.

From the above brood-mutation pattern, and from previous considerations as regards the speed of utilization of the stages of the germ line in our standard brood technique, the following conclusion emerges. The alkyl methanesulphonates are most active in the later stages of spermatogenesis (sperm and latest spermatids); the response decreases the earlier the stage of germ cell differentiation, almost reaching the control level among the earliest germ cells, the spermatogonia.

The yield of the sex-linked recessive visibles in the successive broods under the effect of the alkyl methanesulphonates follows essentially the same trend as the lethals (Table 1). Likewise, the sperm

groups in water expressed as $k \times 10^{-3}$ sec.$^{-1}$ at 37° C. are: 164·8 for the ethyl ester, as compared to 2·4 for the chloroethyl ester, giving a ratio of 68·7 : 1. Expressed in another way, nearly 27 per cent of ethyl methanesulphonate is hydrolysed in water at 37° in 5½ hr., whereas only 20 per cent of the chloroethyl ester is hydrolysed under the same conditions in 10 days; the chlorine atom is not appreciably hydrolysed under the above conditions. While it is fully realized that in vivo the relative reactivity of the two sulphonates may be different, due to differential excretion, detoxication and other biochemical factors, yet it is virtually certain that 2-chloroethyl methanesulphonate is retained in the living tissue in an active (unhydrolysed) state for much longer than ethyl methanesulphonate.

Table 1. Sex-linked Recessive Visibles and Lethals Induced in the Successive Broods under the Effect of the Ethyl and Methyl Esters of Methanesulphonic Acid

Compound	Ethyl methanesulphonate					Methyl methanesulphonate									
Concentration injected	$1 \cdot 61 \times 10^{-3}$ M					$0 \cdot 25 \times 10^{-3}$ M					$0 \cdot 45 \times 10^{-3}$ M				
Brood	Chromosomes tested	Visibles No.	%	Lethals No.	%	Chromosomes tested	Visibles No.	%	Lethals No.	%	Chromosomes tested	Visibles No.	%	Lethals No.	%
1	485	23	5·0	88	18·3	502	13	2·6	32	6·3	435	21	4·6	53	11·6
2	350	13	3·7	84	14·6	473	8	1·7	27	5·7	695	22	2·4	53	10·5
3	301	17	5·6	47	15·6	504	2	0·4	26	5·2	515	13	2·2	52	7
4	333	9	2·3	45	12·0	487	2	0·4	19	3·9	442	9	1·9	44	9·1
5	318	—	—	1	0·3	451	—	—	10	2·1	429	1	0·2	15	1·0
6	345	1	0·3	2	0·6	526	—	—	2	0·3	326	—	—	2	0·6
7	324	—	—	3	0·9	511	—	—	1	0·2					
Total	2,431	63	2·5	231	9·6	3,541	25	0·7	117	3·3	3,433	60	1·7	228	6·6
Ratio visibles/lethals	0·27					0·21					0·26				

and latest spermatids give the highest yield of visibles. The overall ratio of visibles to lethals shows some variation among the various sulphonates. For both the mono-functional methanesulphonates (ethyl and methyl esters) this ratio is 0·26 ± 0·01 (Table 1) and is, therefore, significantly higher than for the di-(methanesulphonoxy) alkanes, where it is 0·12 ± 0·02 (see ref. 5). It is to be noted that this ratio, even for the mono-methanesulphonates, is lower than that for 2-chloroethyl methanesulphonate (C.B. 1506), which gives an overall ratio of 0·42 ± 0·02 (see ref. 6).

Mechanism of Mutagenesis under the Alkyl Methanesulphonates

In a recent communication[4] we directed attention to the fact that for 2-chloroethyl methanesulphonate, the highest mutagenic response was in the very early germ cells (utilized more than 12 days after treatment), and the lowest was in mature sperm. This is the reverse situation to that produced with ethyl methanesulphonate, or indeed any other mono- or di-methanesulphonate so far investigated in our laboratory, where the maximal effect is on the later germ cells. By way of illustration we have given in Fig. 2 the actual experimental curve for the brood/mutation-rate under the effect of ethyl methanesulphonate (curve A, based on Table 1) and compared it to that for roughly the same injected molar concentration of the chloroethyl ester (curve D, based on the previously published data[7]).

From the chemical point of view, both ethyl and 2-chloroethyl methanesulphonate are potential alkylating agents, but the chemical reactivity of the sulphonoxy group is much higher in the former compound. The data of Davis and Ross[7] show that in vitro the hydrolysis constants for the sulphonoxy
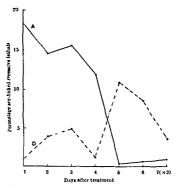

Fig 2. Comparison of the brood-mutation patterns (sex-linked recessive lethal rate) for ethyl methanesulphonate (curve A, conc $1 \cdot 6 \times 10^{-3}$ M, Table 1) and 2-chloroethyl methanesulphonate (curve D, conc $1 \cdot 4 \times 10^{-3}$ M, data in ref. 7)

The above difference in the chemical reactivity of the two sulphonates may explain some of the differences in their brood-mutation patterns (Fig. 2). The much higher mutation-rate induced in the sperm by ethyl methanesulphonate (as compared with the

AUTONOMOUS ACTION OF LETHAL MUTATIONS INDUCED IN THE GERM CELLS OF DROSOPHILA MELANOGASTER BY 2-CHLORO-ETHYL METHANESULPHONATE

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IN young *Drosophila* males the sperm to be utilized for successive broods of offspring comes from successively earlier stages of spermatogenesis in the testis, from mature sperm to early spermatogonia. Following treatment with a mutagen, the pattern of effect measured in successive broods will be a reflexion of the sensitivity of the various germ cell stages to the action of the mutagen.

The mutagenic properties of 2-chloroethyl methane-sulphonate (C.B. 1506) have been studied by Fahmy and Fahmy[1], who found that the ratio of sex-linked recessive visibles to lethals rises in the later broods, from 0·35 in broods utilizing sperm from post-meiotic cells to 0·6–1·0 in broods utilizing sperm from pre-meiotic cells at the time of treatment. Three possible explanations of the phenomenon may be listed.

(1) The mutagen may have some specific action on the visible loci concerned. This presupposes some common chemical properties of the visible loci as a whole as compared with the lethals as a whole and also leaves unexplained the difference between pre- and post-meiotic cells.

(2) The mutagen may induce more 'point mutations' in the pre-meiotic cells. This would produce an increase in the visible to lethal ratio, since in larger areas of chromosomal damage, the locus with the earliest action[2] would be the definitive one, masking the presence of others, in this case, visibles.

(3) Essentially the same proportions of the different mutations may be produced in all cells, but some of the lethals induced in the pre-meiotic cells may act autonomously, killing the cell before it produces sperm, thereby increasing the observed proportion of visibles.

This present investigation was undertaken to examine the last possibility, that the increase in the ratio of visibles to lethals was produced by adverse selection in the germ line against lethals functioning autonomously.

Approximately 0·2 µl. of a 0·2 per cent solution of C.B. 1506 in 0·4 per cent saline was injected into the abdomen of young *Drosophila* males (Oregon-K stock). [...] were [...] on of muta-tions. Lethals were scored as cultures containing no non-Muller-5 males at all in the F_3 and confirmatory F_3 for the sex-linked data, and no $Cy+/L+$ flies in the F_3 and F_4 for the 2nd chromosome data. Visible mutations were scored in the sex-linked experiment only, and scored as mutations producing any non-Muller-5 males which showed any deviation from the Oregon-K phenotype. 'Semi-lethals' which produced a marked reduction in the numbers of wild-type

males in the F_3, but with no phenotypic deviation, were not classed as visibles nor included in the lethal data.

The results are shown in Table 1 and represented graphically in Fig. 1. Broods I–IV and VI–IX are taken together, since the comparisons to be made are between post-meiotic mutations I–IV, and pre-meiotic mutations VI–IX. Brood V is not included in either since overlapping may occur here. The yield of 2nd chromosome lethals is approximately double that of sex-linked lethals for the first four broods. A two by four contingency table of the sex-linked and 2nd chromosome lethals in the first four broods gives a value of $\chi^2 = 2·9$; $\nu = 2$; $P = 0·25$; thus there is no indication of heterogeneity through-
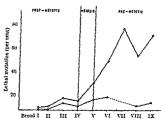

Fig. 1. The brood pattern of X-chromosome lethals (△), and second chromosome lethals (○), induced by 2-chloroethyl methane-sulphonate

out these broods. For broods I–IX (except VII) $\chi^2 = 16·5$; $\nu = 6$; $P = 0·01$. This indicates a considerable amount of heterogeneity in the results which must be due to the increase in the ratio of autosomal to sex-linked lethals in the later broods, the value 5·3 for broods VI, VIII and IX being well outside 3s for broods I–IV, the ratio being 1·9 ± 0·33 (Table 1).

The exceptionally low incidence of visibles in brood I in the present experiment is probably not significant. [...] of many lethal mutations [...]

fate of cells homozygous or hemizygous for a lethal mutation, arising by somatic crossing-over or non-disjunction in visible heterozygotes, and found that even single cells surrounded by normal tissue die. Similarly, Gloor[7] demonstrated that some lethal factors function autonomously in male germ cells transplanted into genetically normal hosts, the germ cells not developing beyond the stage they normally support. The second is also untenable, since it more [...]

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ame low chemical reactivity (same hydrolysis-rate) as the chlorethyl ester, but would not be expected to produce in vivo a highly reactive 'mustard'. The mutation peak at the fifth brood which occurred with the chlorethyl ester, and which has been attributed to a strong secondary mutagen (presumably a 'mustard') would not, therefore, occur with the fluorethyl ester. The genetical results so far obtained (to be detailed elsewhere) conformed with the biochemical expectation. Under the effect of fluorethyl methanesulphonate the mutation-rate increased for the first three broods (reaching roughly half the rate induced by the same molar concentration of the chlorethyl ester) and then levelled off into a plateau until the seventh brood. There can scarcely be any doubt, therefore, that the high mutagenic response of the early germ cells under the effect of 2-chlorethyl methane sulphonate is an indication of the formation of a secondary, highly active mutagen. An attractive property of this mutagen is its potency in the induction of 'visibles'. It is, therefore, hoped that its successful identification and synthesis might well provide us with an agent highly selective for the induction of this class of mutation.

[1] Bird, M. J., and Fahmy, O. G., *Proc. Roy. Soc.*, B, 140, 556 (1953).
[2] Fahmy, O. G., and Fahmy, M. J., *J. Genet.*, 52, 603 (1954).
[3] Fahmy, O. G., and Fahmy, M. J., *J. Genet.*, 53, 563 (1955).
[4] Fahmy, O. G., and Fahmy, M. J., B.E.C.C. Annual Report, 30, 39 (1952).
[5] Fahmy, O. G., and Fahmy, M. J., *J. Genet.*, 54, 146 (1956).
[6] Fahmy, O. G., and Fahmy, M. J., *Nature*, 177, 996 (1956).
[7] Davis, W., and Ross, W. C. J., personal communication
[8] Roberts, J. J., and Warwick, G. P., *Nature*, [179, 1181 (1957)]

(901)

Tumor Progression

GUEST EDITORIAL

The belief that, once a neoplasm is initiated, its characters are fixed for all time is losing ground but still lingers. The analysis of successive steps in neoplasia has been slow and difficult. The significance of many lesions reported as "precancerous" is uncertain, and the wildly disparate assessments of the importance of such conditions as gastric ulcer and cystic mastitis as precursors of cancer are notorious. Although experimental pathologists enjoy the immense advantage of being able to induce tumors at will in a variety of ways, they face two of the same major difficulties that hamper the study of tumor development in man: one is distinguishing between collateral and ... nomenclature of morbid histology by themselves are not good enough. The behavior of tumors under varied conditions and their reactivity or responsiveness to extrinsic stimuli yield clear evidence of progression through a sequence of steps. Progression is defined as the development of a neoplasm by way of permanent irreversible qualitative change in one or more of its characters.

The first step in neoplasia is a permanent qualitative change or progression; it does not necessarily entail proliferation of the cells or distinctive histological change, but the reactivity of the cells is altered. As early investigators found, the tarred skin of mice changes irrevocably long before neoplasia is distinguishable macroscopically or microscopically. In the tarred skin of rabbits latent tumor cells—cells in a subthreshold neoplastic state, according to Rous—come into being quickly, but they do not multiply unless stimulated by promoting factors which by themselves are not carcinogenic. The tumors regress when the stimulus is withheld and recur when it is restored. The latent tumor cells have a permanently altered reactivity; they respond to noncarcinogenic stimuli by neoplastic growth as normal cells do not. The resulting tumors are *conditional*, being dependent for their growth on external stimulation. Some of them are papillomas and are benign by histological criteria, but others, named carcinomatoids, are invasive and look like carcinomas. The character of conditional, dependent growth cuts across histological classifications. As *Huggins* showed for cancer of the prostate in man, some tumors with all the marks of malignant carcinomas are nevertheless responsive to, or dependent upon, hormonal stimulation. Tumors induced experimentally by hormones are often conditional, and so are some mammary tumors of mice. The responsiveness of these tumors to hormones and of chemically induced skin tumors to varied stimuli provides valuable indicators of progression, since the responsiveness usually changes and disappears. General principles of progression inferred from the study of natural and induced tumors in animals seem widely applicable to neoplasia in man. Two or three of them are especially relevant to current approaches to the management of human cancer.

Different characters of a tumor undergo progression independently of one another. Some mammary tumors of mice are conspicuously responsive to the hormonal changes accompanying reproduction; they grow only during pregnancy and regress after parturition. Progression to an unresponsive state of continuous growth independent of reproductive activity occurs without progression in growth rate. Conversely, progression in growth rate can occur without change in responsiveness. ...

independent progression of characters leads to the inference that the structure and behavior of tumors are determined by numerous unit characters which, within wide limits, are *independent-*

Table 1 A COMPARISON OF THE INDUCTION OF SEX-LINKED RECESSIVE MUTATIONS (A) AND 2ND CHROMOSOME RECESSIVE LETHALS (B), BY C.B. 1506

	Days	4	2	3	3	3	3	3	3	
A	Brood	I	II	III	IV	V	VI	VII	VIII	IX
	Total chromosomes tested	1,501	406	249	117	161	135	9	166*	53
	Total lethals	16	2	16	4	16	18	—	6	4
	X_1 Lethals (per cent)	1.1	0.5	6.5	3.4	10.0	12.6	—	3.6	6.9
	Weighted mean	$M_1 = 3.6$; $\sigma_1 = 0.41$					9.6			
	Total visibles	1	1	7	2	7	10	—	9	—
	Visibles/lethals	0.06	0.5	0.4	0.5	0.4	0.63	—	1.5	—
	Total visibles/lethals	0.20					0.73			
B	Total chromosomes tested	707	282	181	69	110	81	15	80*	34
	Total lethals	17	10	21	6	29	39	12	44	25
	X_2 Lethals (per cent)	2.4	3.5	11.6	8.7	26.4	48.0	80.0	53.7	73.6
	Weighted mean	$M_1 = 6.7$, $\sigma_1 = 0.76$					50.6			
	Ratio II/X	2.2	7.0	1.8	2.6	2.6	3.8	14.3	10.7	
		1.9 ±f = 0.11					5.3			

* One group of 31 F_2 cultures (A) and 25 F_2 cultures (B) from a single male which was infertile in all other broods has been eliminated.

$$\dagger Z = \frac{X_1}{X_2}; \quad n^2 = \frac{M_1^2}{M_2^2}(v_1^2 - 2r\,v_1\,v_2 + v_2^2); \quad v_1 = \frac{\sigma_1}{M_1}; \quad r = 0.87.$$

'point mutations' were produced in the later broods, the same proportion of autosomal and sex-linked lethals should be produced throughout, and this is clearly not so. The third explanation fits the observations. Developing spermatids and spermatozoa seem to be protected against the harmful effects of quite drastic genetical damage, since extensive chromosomal aberrations which later produce dominant lethality persist in sperm which is still capable of fertilization. Therefore, the proportions of these lethal mutations and sex-linked visibles detected in the late germ cells are a good estimate of the actual proportion produced in the cells themselves. The results show a striking increase in the yield of autosomal recessive-lethals and sex-linked recessive visibles against sex-linked recessive lethals in late broods. The former mutations are both ineffectual when induced in premeiotic cells ; the autosomal lethals exist in the form of viable heterozygotes, the visibles are by definition non-lethal ; whereas sex-linked lethals in the hemizygous state may be eliminated by autonomous action. Since there is no evidence that the above classes of mutations are produced in different proportions in different chromosomes, throughout the stages of the germ line, it must be concluded that the divergence in the relative yields of autosomal lethals and sex-linked visibles from sex-linked lethals is most probably due to adverse selection against the latter by autonomy.

I wish to thank Dr. O. G. Fahmy for his interest in this work This investigation has been supported by grants to the Chester Beatty Institute (Institute of Cancer Research, Royal Marsden Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

[1] Fahmy, O. G , and Fahmy, M J , Nature, 177, 996 (1956)
[2] Hadorn , E , "Adv In Genetics", 4, 53 (1951)
[3] Auerbach, C , Z Vererbungslehre, 88, 113 (1954)
[4] Ephrussi, B , Proc U S. Nat Acad Sci , 20, 420 (1934)
[5] Demerec, M , Amer Nat., 68, 165 (1934)
[6] Stern, C , Proc U S Nat Acad Sci , 21, 374 (1935).
[7] Gloor, H , Rev. Suisse Zool , 50, 339 (1943).

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Changes in the Male Accessory Sex Organs, and in the Testes, Adrenals and Pituitary, Associated with the Growth of Animal Tumours

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Taken together, the diverse effects of tumours upon the organism of the host comprise a topic which is as old as cancer research itself, but it is one of which the fundamental importance has perhaps

We are familiar with specific effects on the host economy brought about by a large variety of so-called functioning tumours, as, for example, of the testis and ovary, the adrenal medulla and cortex, the anterior pituitary, the thyroid, the islet cells of the pancreas, and the argentaffin tissues,—to quote but a few. In such cases the effects are due to the retention, in an unchanged or sometimes modified form, of some secretory function of the tissue of origin. As such they are perhaps not too difficult to comprehend, and have perhaps only special or particular interest, with no real general significance for the cancer problem at large. At the other extreme, we are equally aware of the non-specific contribution to the clinical state of cancer cachexia, brought about by such factors as invasion and destruction of vital structures, anatomical obstruction, and secondary infection. There remains, however, the question whether tumours in general, and as a class, may not produce much more specific alterations in the nutrition and endocrinology of the host—whether by depletion or through the release of some toxic principle; and, in particular, whether cancer cachexia may include some element more specific and elusive—and hence more important—than the somewhat incidental and mechanical factors already

ly variable and liable to independent progression and which can be combined in a variety of ways. The response to treatment depends on such characters. The term "malignancy" becomes dangerous if understood to imply an indivisible entity or quality. Many characters contribute to malignant behavior. The classical signs of malignant tumor combine variously with one another and with varied degrees of responsiveness to extrinsic stimuli. A tumor may show one or all of the orthodox criteria of malignancy and yet be conditional or responsive and amenable to treatment, whereas a tumor more "benign" histologically may be intractable. The histological distinction between "benign" and "malignant" is no longer a sufficient guide for therapy; responsiveness to hormones or chemical agents, not necessarily correlated with histological structure or marks of malignancy, is becoming crucially important. The responsiveness is liable to progression which ultimately frustrates all or most attempts to control cancer by methods which fall short of complete eradication.

The term "precancerous" has been applied to lesions of at least two different kinds, subject to different modes of progression. Some lesions almost inevitably eventuate in cancer; progression advances along a predetermined path to a predictable end-point. The characters of a malignant tumor develop by progression which is characteristically gradual and inexorable. This mode of progression operates in many experimental tumors and probably in many tumors of man, for example, those papillomas of the bladder that are recognizable from an early localized stage as destined to end as carcinomas. A different form of progression operates in "precancerous" lesions whose fate is not a foregone conclusion, advancement to carcinoma being erratic and unpredictable. Progression entails a switch of development into a new path rather than acceleration along the old path. New, more dangerous properties and, in effect, a new tumor come into being. This type of progression, characteristically abrupt and unpredictable, occurs in mammary tumors and chemically induced skin tumors in mice and in the development of carcinoma from intestinal papillary adenoma or familial polyposis in man. The differentiation of this type of progression is important because, although the experimental evidence is inconclusive, there is a strong possibility

that extrinsic stimuli may decisively control progression.

The term "carcinoma *in situ*," applied especially to localized lesions of the uterine cervix in women, seems to be creeping into use for comparable lesions of the lung and breast. The mode of progression of the lesions to invasive carcinoma needs clarification. The recognition of these early steps in neoplasia is important and welcome, but the terminology is unfortunate. It carries the implication that the early lesions are carcinomas that have not yet invaded and differ from invasive carcinoma not at all in the characters of the cells but only in their spatial extension. This is probably untrue. According to present estimates, carcinoma *in situ* precedes invasive carcinoma by about 10 years. It is not easily credible that the lesions develop continuously without qualitative change throughout this period. More probably carcinoma *in situ* is not carcinoma at all but an early, possibly hormone-responsive, stage of neoplasia that acquires invasiveness and other malignant characters by progression at a time close to the onset of clinical cancer.

Emancipation from obsolete restrictive terminologies and habits of thought is a prerequisite for the best direction of the renewed interest in the natural history of tumors and their progression through consecutive stages variously responsive to extrinsic stimuli and variously amenable to treatment. Neoplasia begins before it is recognizable visually. Some precancerous lesions are in fact neoplastic; their progression to carcinoma is irreversibly determined, although histological criteria of neoplasia are not fulfilled. The antitheses between "benign" and "malignant" and between "dependent" and "autonomous" are too rigid. The unit characters of tumors are far more diversely assorted than these terms allow; "malignancy" and "dependency" are infinitely graded and variously combined. It is possible, as long suspected by some, that the characters that most decisively govern the outcome of neoplasia and its response to treatment are as yet unknown.

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ably a factor in stimulating the sexual urge in the male. Secretory activity is hormonally regulated, and castration, treatment with œstrogen, or feeding on a diet deficient in the B vitamins, all induce marked atrophy, which can, however, be reversed by the male sex hormone (5, 6). The seminal vesicles are also of unique biochemical interest, witness the studies of *Mann* (7), who has, for example, demonstrated, in the boar, the secretion of inositol by the same accessory gland as fructose and citric acid (8). These glands are also of some interest as being only rarely the seat of malignant disease (9).

Table 1
Weights (g) in normal rats

Carcase	Seminal vesicles	Prostate
336	0.750	0.530
235	0.460	0.195
228	0 666	0 290
220	0.457	0.270
215	0.396	0.287
190	0.292	0.185
175	0.284	0.273

Table 1 shows an entirely representative relationship between the weights of the carcase, seminal vesicle (and prostate) in normal rats of the inbred albino colony which has been used throughout in these experiments. With these may be contrasted the correspond-

Table 2
Weights (g) in rats bearing the Walker carcinoma 256

Tumour	Seminal vesicles	Prostate	Carcase
95	0.063	0.066	190
95	0 093	0.041	170
90	0.089	0.105	200
90	0.080	0.060	188
86	0.200	0.155	224
65	0.045	0.078	165
58	0 305	0.170	340
55	0.048	0.080	115
55	0.084	0.140	140
54	0.105	–	152
54	0.140	0.100	204
45	0 193	0.145	310
28	0.048	0.082	160
25	0.345	0.200	226
15	0.154	0.180	180
11	0.105	–	192
10	0.325	0.336	240
5	0.395	0.120	230

mentioned. The past few years have seen an increased receptiveness to such possibilities, and their very existence is ample justification for further search, in view of the bearing—perhaps more than theoretical—which the results might possess for cancer control and prevention.

The present paper records an observation made several years ago, of which brief accounts have already been given in the Annual Reports of the British Empire Cancer Campaign (1), namely, of a profound degree of atrophy of the seminal vesicles in rats bearing a variety of tumours both transplanted and induced, an effect also accompanied by alterations in the adrenals and pituitary. The observation was incidental and unexpected, and was made in a curious fashion. During experiments to measure the inhibitory action upon the Walker rat carcinoma 256 of a number of potent cytostatic agents belonging to a series of so-called aromatic nitrogen mustards (2), it was noted that the weights of the seminal vesicles in the treated series were greatly in excess of those of the controls. A stimulatory effect seemed improbable, and on comparing the seminal vesicles in rats (a) bearing the Walker tumour, (b) implanted with the Walker tumour, the growth of which had however been greatly retarded by treatment with these cytostatic agents, and (c) without tumours, it at once became obvious that the explanation lay in a severe atrophy of the vesicles concomitant with tumour growth (group a). So far as we are aware, when this observation was made it had not previously been reported. Meantime, however, *Begg* and *Stewart* (3), and *Begg* (4) have recorded that the seminal vesicles of tumour-bearing rats are smaller than those of controls, and we understand that the phenomenon has also been noted by *O. Mühlbock* of Amsterdam (personal communication). Before proceeding to describe the effect itself, a brief account may be given of the structure and function of these organs. The seminal vesicles in rodents are elongated, hollow bodies with an irregular branched lumen and numerous outpocketings, and arise as evaginations of the *ductus deferens* near its termination. Histologically, the structure consists of secretory epithelial cells in numerous folds, and it is these cells—highly susceptible to great variation as their appearance is dependent upon age and many functional influences—which provide part of the seminal fluid. The essential structure is a round, cuboidal or low columnar epithelium with basal cells which, when

vided with a plexus of nerve fibres and with small sympathetic ganglia. The secretion produced by the epithelial lining accumulates in the cavity of the vesicle, and the resulting engorgement is prob-

Fig. 1. Glandular epithelium of the seminal vesicle of a control rat, showing the folded mucosa and abundant secretion. ×600.

Fig. 2. Atrophic epithelium of the seminal vesicle of a rat bearing the Jensen sarcoma. Note (cf. Fig. 1) the absence of secretion. ×600.

... or not similar changes accompany the growth of
other animal tumours, measurements were carried out in animals
...bvious interest to deter-

Table 3

Weights (g) in tumour-bearing rats and mice

Tumour	Tumour weight	Seminal vesicles	Prostate	Carcase
Jensen rat sarcoma	148	0.095	0.180	
	112	0.060	0.095	223
	95	0.095	0.130	173
	38	0.300	0.265	170
R 48 rat sarcoma	64	0.110	0.195	235
	60	0 050	0.120	162
	41	0.160	0.190	156
	40	0.100	0.110	199
	24	0.130	0.205	155
	19	0.215	0.200	162
Primary 3.4-benzpyrene-induced rat sarcomas	340	0.868	–	184
	320	0.650	–	370
	260	0.668	–	370
	240	0.294	–	470
	200	0.710	–	290
	190	0.876	–	320
	145	1.520	–	356
	105	1.080	–	418
	104	1.425	–	440
	80	1.480	–	430
	0	1.661	–	375
Crocker mouse sarcoma 180	Average tumour weight 8.6 g	0.179	–	555
		0.160	–	
		0.147	–	Average carcase weight 33 g
		0.144	–	
		0.126	–	
		0.115	–	
		0.101	–	
Controls to Crocker sarcoma	0	0.362	–	
	0	0.355	–	
	0	0.349	–	
	0	0.320	–	
	0	0 293	–	
	0	0.276	–	Average carcase weight 40 g
	0	0.281	–	
	0	0.251	–	
	0	0.248	–	
	0	0.212	–	

tumours, revealed in the latter case conspicuous atrophy, the epithelium having changed to a low non-secretory type, with marked increase in the fibro-muscular stroma (Figs. 1 and 2).

Additional experiments were carried out to observe the effect of surgical removal of the tumour on the behaviour of the seminal vesicles. As an example, a Walker graft in a 10-week old rat weighing 120 g was excised when the tumour weighed approximately 12 g. At laparotomy, the seminal vesicles were atrophic, contained little fluid, and measured 16 mm (Fig. 3) The animal remained in good condition, and on its sacrifice one month after tumour removal the vesicles were now seen to have the normal macroscopic appearance, being distended with fluid and measuring 21 mm (Fig. 4).

Begg and Stewart (3) had noted in tumour-bearing rats some atrophy not only of the seminal vesicles and prostate, but also of the testes. In the present experiments, while mild degrees of testicular atrophy could be detected in individual tumour-bearing animals, there seemed to be no general trend in this direction, as is apparent from Table 4. Since the seminal vesicles are, however,

Table 4
Weights (g) of the testes in normal and tumour-bearing rats

Tumour	Tumour weight	Testes weight	Carcase weight
Normal controls	0	3 670	336
	0	2.859	220
	0	3.049	215
	0	2.955	190
	0	2.788	175
Jensen rat sarcoma	148	2.410	223
	112	2.365	173
	95	2.780	170
	38	3.280	235
R 48 rat sarcoma	64	2 630	162
	60	2.600	156
	41	2.765	199
	40	2.215	155
	24	2.925	162
	19	3.100	184
Walker rat carcinoma 256	95	2.518	170
	95	3.049	190
	90	3.153	200
	65	2.595	165
	55	2.600	115
	55	2 230	140
	15	2.695	180
	10	3.222	240
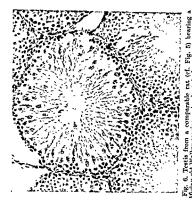
bearing the Jensen rat sarcoma, a transplantable rat sarcoma induced by an aromatic nitrogen mustard (the bis-chloroethyl derivative of 2-naphthylamine [R 48]), primary rat sarcomata induced by 3,4-benzpyrene, and the Crocker mouse sarcoma 180: that the same trend obtains in these cases also, is clearly shown in Table 3. Histological comparison of the seminal vesicles of normal rats and of those of the same age group, but bearing transplantable

Fig. 3. The seminal vesicles in a rat bearing the Walker carcinoma (at laparotomy). ×4.

Fig. 4. The seminal vesicles in the same rat (Fig. 3) one month after removal of the tumour. The macroscopic appearance is normal. ×4.

Fig. 6. Testis from a comparable rat (cf. Fig. 5) bearing a 16-day-old Walker carcinoma implant, in frozen section stained with Sudan IV.

Note the considerable increase in Sertoli cell lipids.

Fig. 5. Testis from a normal control rat, in frozen section stained with Sudan IV.

specially sensitive to nutritional and endocrine changes, and especially to androgen depletion, it was necessary to look more closely for alterations in the structure of the testis, not necessarily shown by any change in organ weight. Accordingly, cytological studies were made of frozen sections of testes from both normal and tumour-bearing rats, stained with Sudan IV. At once it became apparent that testes from the tumour-bearing animals showed an incr~~~~~~
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production of spermatozoa, being released into the lumina of the tubules with the mature sperm, but *retained within the Sertoli cells in the absence of spermatogenesis.* This present observation can therefore be taken as suggestive of an interference with testicular function in tumour-bearing animals, not however immediately or necessarily reflected in any change of organ size or weight.

Coincident with the alterations already described, marked changes were observed in the adrenal glands of tumour-bearing animals, similar to those already reported by others. Table 5

Table 5

Adrenal weights (g) in normal male rats and rats bearing the Walker carcinoma 256

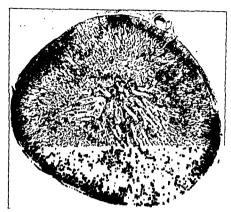| Tumour weight	Weight of adrenals	Carcase weight
Normal controls	0.030	235
	0.050	220
	0.064	215
	0 050	190
	0 049	175
95	0.144	190
95	0.316	170
90	0.311	200
90	0.155	188
86	0.095	224
65	0.100	165
58	0.050	340
55	0.251	115
55	0.103	140
54	0.092	152
54	0 085	204
45	0.055	310
28	0.082	160
25	0.050	226
15	0.075	180
11	0 054	192
10	0.059	240
5	0.045	230

Fig. 9. Adrenal gland from rat bearing the Walker carcinoma. Note pronounced hyperæmia in both medulla and cortex (cf. Fig. 8). ×20.

Fig. 10. Transverse section of the pituitary gland of a normal rat, showing the *pars anterior*, the *pars intermedia* and the posterior lobe. ×10.

Finally, attention has been given to alterations in the pituitary gland itself. When related to body weight, the weight of the pituitary did not appear significantly to vary as between normal and tumour-bearing rats (Table 6). However, the pituitary glands of the latter frequently show histological changes. mainly involving

Fig. 7. Illustrating the macroscopic difference in adrenal glands from control rats (on left, two) and those from rats bearing the Walker carcinoma (on right, four).

Fig. 8. Adrenal gland from control rat (cf. Fig. 9). ×20.

illustrates the considerable increase in the weights of these organs in male rats carrying the Walker carcinoma—most pronounced perhaps in the younger animals bearing the largest tumours. Histologically, this increase in size and weight (Fig. 7) appears largely

ectomised animals, suggested its possible mediation by stimulation of pituitary adrenotrophic hormone by the products of tumour breakdown. *Kropp* and *McEwen* (13) draw attention to the great lability in the histology of the adrenal cortex of rats bearing the Walker tumour, the relative abundance of cell types (eosinophilic, basophilic, and giant cells) apparently not being related to tumour size.
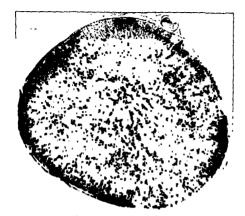
Fig. 9. Adrenal gland from rat bearing the Walker carcinoma. Note pronounced hyperæmia in both medulla and cortex (cf. Fig. 8). ×20.

Fig. 10. Transverse section of the pituitary gland of a normal rat, showing the *pars anterior*, the *pars intermedia* and the posterior lobe. ×10.

Finally, attention has been given to alterations in the pituitary gland itself. When related to body weight, the weight of the pituitary did not appear significantly to vary as between normal and tumour-bearing rats (Table 6). However, the pituitary glands of the latter frequently show histological changes, mainly involving

Table 6

Pituitary weights (g) in normal and tumour-bearing rats

Normal

Carcase	256	252	250	250	248	248	246
Pituitary	0.006	0.009	0.006	0.008	0.009	0.007	0.006
Carcase	242	242	242	240	240	240	238
Pituitary	0.007	0.006	0.006	0.006	0.008	0.008	0.006
Carcase	236	236	236	235	235	235	232
Pituitary	0.008	0.007	0.008	0.006	0.006	0.008	0.006
Carcase	232	230	228	226	235	235	232
Pituitary	0.008	0.006	0.006	0.007	0.006	0.007	0.006
Carcase	224	223	221	220	225	225	225
Pituitary	0.007	0.007	0.006	0.008	0.008	0.007	0.006
Carcase	214	210	210	206	220	220	225
Pituitary	0.007	0.006	0.006	0.006	0.007	0.007	0.007
Carcase					205	205	220	215
Pituitary					0.007	0.005	0.007	0.008
Carcase							202	198
Pituitary							0.007	0.006

Walker carcinoma

Tumour	87	74	68	65	63	62	61
Carcase	256	251	267	255	272	286	285
Pituitary	0.008	0.008	0.009	0.009	0.009	0.008	0.008
Tumour	57	56	48	48	47	45	44
Carcase	251	266	280	267	276	255	240
Pituitary	0.008	0.008	0.008	0.009	0.008	0.008	0.008
Tumour	36	32	22	14	13	10	9
Carcase	259	248	267	251	247	290	290
Pituitary	0.008	0.008	0.008	0.008	0.008	0.009	0.009

Jensen sarcoma

Tumour	148	112	95	38
Carcase	223	173	170	235
Pituitary	0.007	0.004	0.006	0.006

Sarcoma R 48

Tumour	64	60	41	40	24	19
Carcase	162	156	199	155	162	184
Pituitary	0.007	0.007	0.007	0.006	0.004	0.006

the pars anterior but by no means constant. When they do occur, the anterior lobe becomes enlarged, with an increase in the number of chromophobe cells and a marked decrease in the number of acidophiles (Figs. 10–13). No changes were noted in the pars posterior except for masses of colloid in the cleft between the pars intermedia and the pars anterior.

Endocrine and nutritional aspects

Atrophy of the seminal vesicles is a sequel to castration, and the epithelial changes so produced are reversed on administration of androgen (5) (also Moore, Hughes, and Gallagher [14]). Similar

Table 7

Influence of testosterone propionate (9 daily doses of 25 mg)
upon the weight of the seminal vesicles in rats
bearing the Walker carcinoma (cf. Table 2)

	Weight (g)	
Tumour	Seminal vesicles	Carcase
64	0.9	195
60	0.9	195
59	1.0	200
54	1.0	170
32	1.6	190
24	1.2	235
7	1.1	215
3	1.5	220
1	1.3	205
0	1.5	190

recorded the response of inbred strains of mice to androgen, as
measured by the mitotic rate in the seminal vesicle epithelium.

As for testosterone propionate, similar experiments were con-
ducted to test the action of serum gonadotrophin, which likewise

Table 8

Influence of serum gonadotrophin
(British Drug Houses "Serogan". 13 daily doses of
1000 international units)
upon the weight of the seminal vesicles in rats bearing the
Walker carcinoma (cf. Tables 2 and 7)

	Weight (g)	
Tumour	Seminal vesicles	Carcase
96	1.3	190
67	1.2	200
56	1.8	190
55	1.5	180
53	1.4	205
42	1.3	195
38	1.3	180
35	1.3	170
34	0.8	155
25	1.3	195

compare Figs. 14 and 16). This result supports the observation of
Begg (4) that while the seminal vesicles are sensitive to atrophy in
tumour-bearing rats, they are still responsive to exogenous gonado-
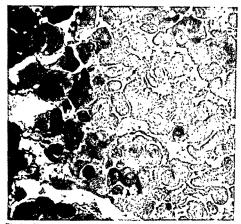trophin; and also, in part, the suggestion made by *Begg* and *Stewart*

Fig. 13. Histology of the *pars anterior* of the specimen shown in Fig. 12 (cf. Fig. 11). Note increase in chromophobe cells and decrease in acidophils.

production through adrenal hypertrophy, although in this event a more pronounced effect upon testicular weight might also be expected. Experiments were however undertaken to test the effect, in rats bearing the Walker carcinoma, of simultaneous treatment with testosterone propionate. A typical result is shown in Table 7, from which it appears that the seminal vesicles underwent not atrophy but a *considerable hypertrophy—still, however, probably* less in extent in those animals bearing the largest tumours. The resulting stimulation of secretion is seen by comparing Figs. 14 and 15. The effects of testosterone propionate on the seminal vesicles of the rat had already been studied by *Porter* and *Melampy* (16), measuring the resultant increases (in castrates) of wet weight, nitrogen content, secretion, alkaline, and acid phosphatases, and catheptic activity. *Melampy* and *Cavazos* (17) also found that atrophy of the seminal vesicles in pantothenate-deficient mice is responsive to the male sex hormone and possibly due to a lack of pituitary gonadotrophin. Lastly, *Chai* and *Mendelsohn* (18) have

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Fig. 16. Seminal vesicle from rat bearing the Walker carcinoma and treated with serum gonadotrophin. Note the abundant secretion (cf. Fig. 14). The lateral prostate is seen adjacent. ×10.

Fig. 17. Seminal vesicle from rat bearing the Walker carcinoma. Note inhibition of secretion and increase in the fibromuscular stroma. ×9.
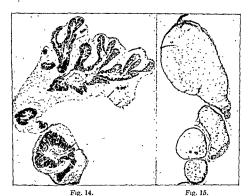
Fig. 14.   Fig. 15.

Fig. 14. Atrophic seminal vesicle from rat bearing the Walker carcinoma.
Note inhibition of secretion and increase of the fibromuscular stroma × 10.

Fig. 15. Seminal vesicle from rat bearing the Walker carcinoma and treated
with testosterone propionate. Note the abundant secretion and decrease
in stroma (cf. Fig. 14). × 10.

(3) that the presence of a tumour may produce a deficiency of
gonadotrophic hormones. It is of interest that *Greenberg* and
Ershoff (19) found rats deficient in essential fatty acids to exhibit
marked atrophy of the prostate and seminal vesicles, and that
administration of either methyl linoleate or chorionic gonado-
trophin restored these organs to the normal weight.

Lastly, following the observation of *Moore* and *Price* (20), that
implantation of rat pituitaries prevents œstrogen-induced atrophy
of the seminal vesicles, experiments were performed which con-
clusively proved that regular subcutaneous implants into tumour-
bearing hosts of fresh pituitary glands from 10-week-old male
rats (27 whole pituitary implants in 17 days) also prevents atrophy
of the seminal vesicles; the result being similar to that obtained
by treatment with either testosterone propionate or serum gonado-
trophin (cf. Figs. 17–19).

Quite apart from a primarily endocrine mechanism, an additional
hypothesis must be considered, namely, to what extent the tumour
imposes a nutritional deficiency on the host, thereby reducing the

sensitivity of the target organs to the normal levels of hormonal stimulation. The normal size of the testes in contrast with the accessory glands, and an observation that the 17-ketostéroid output of rats bearing the Walker carcinoma does not differ significant-
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are concerned in the response of the male accessory organs to stimulation by androgenic hormone. Of special interest, *Moore* and *Samuels* (21) showed that a diet deficient in vitamin B produced a decrease in the size of the prostate and seminal vesicles of rats *without a corresponding effect on the testes or on spermatogenesis*. The accessory organs could be restored to normal size by injection of androgenic or anterior pituitary hormone. *Lutwak-Mann* and *Mann* (22, 23) have reported similar observations, that the secretory activity of the coagulating glands, prostate and seminal vesicles is greatly reduced in rats kept on a vitamin B- (and B_1-) deficient diet, the activity again being restored to normal by administration of testosterone propionate or chorionic gonadotrophin. Lastly, *Goldsmith, Black,* and *Nigrelli* (24) have shown that deficiency of a single member of the B complex, namely folic acid, can lead to the same result: folic acid antagonists fed for considerable periods to castrate rats produced a markedly decreased response of the accessory glands to testosterone propionate.

In the present work, preliminary experiments to test the influence of dietary supplementation with folic acid (4 mg per rat daily) proved negative, or at the most showed only a slightly less degree of seminal vesicle atrophy than in the absence of supple-

equal groups. Groups I and II were fed a stock diet[1] and Groups III and IV a breeding diet[2] supplemented with 1 g Bemax per rat per day. After three weeks on this regime, groups II and IV were implanted with the Walker carcinoma and the same diets

[1] *Stock diet:* Bread 6 days weekly, milk thrice weekly, rat cake nuts twice weekly, margarine, marmite, cod liver oil, dog biscuits, oats, each once weekly. Fresh water daily.

[2] *Breeding diet:* Mash 6 days weekly, rat cake nuts twice weekly, bread twice weekly, marmite, cabbage, cod liver oil, dog biscuits, oats, each once weekly. Fresh water daily. - *Rat cake nut formula* (%): Fine bran 17.4; ground wheat 17.4, Sussex ground oats 17.4, ground maize 8.7, ground barley 8.7, white fish meal 4.8, meat and bone meal 9.6, dried skimmed milk 14.0, dried yeast 1.2, salt 0.4, cod liver oil 0.4. - *Mash formula* (%): Scraps 50, soaked bread 25, milk powder 6, casein 5, Bemax 4, bran 4, yeast 3, salt 1, cod liver oil 1, chalk 1.
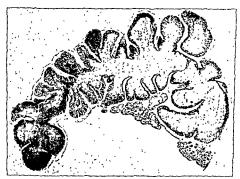
Fig. 18. Seminal vesicle from rat bearing the Walker carcinoma and treated with repeated implantation of fresh homologous pituitary glands (cf. Fig. 17). ×9.

Fig. 19. Seminal vesicle from comparable non-tumour-bearing rat, which had received repeated implantations of fresh homologous pituitary glands (cf. Figs. 17, 18). ×9.

respects but by no means all—to those produced by a diet deficient in protein. There is, however, the further possibility that the alterations in the host are due to the liberation of a specific substance or substances from the tumour itself: in an excellent review of malignant cachexia, *Donovan* (29) expressed himself as more attracted to such a view, than to an explanation based upon depletion alone. In this event, the changes described in the present paper could be induced through a mechanism similar to—or conceivably identical with—that responsible for what *Greenstein* (30) has called one of the established phenomena of cancer, namely, *the depression of liver catalase activity* concomitant with the growth of many tumours in rats and mice. Here, the effect is produced by

material may be present in normal tissues (33), it occurs there to a

protein-like substance also with the capacity markedly to lower the liver catalase in mice, possibly by an interference with enzyme synthesis through binding to iron. In the present work, several experiments were carried out to determine whether the changes in the male accessory glands, and in the adrenals and pituitary, in a tumour-bearing rat, do or do not occur in a normal rat in parabiotic union with it. The results have not been reported in detail since they proved equivocal, but it is of interest that *Lucké, Berwick* and *Zeckwer* (42) were successful in demonstrating that the substance responsible for the depression of liver catalase activity in tumour-bearing animals can so be transferred across a parabiotic union. These experiments, and others designed to seek for the presence of a substance in tumour tissue comparable with that responsible for catalase depression, are being continued. It will also be necessary to relate the present findings to other somatic and biochemical changes occurring in the host at progressive stages of tumour growth—in organ weights and chemical composition (43, 44) with special reference to lipid and water content (45-50); in normal mitotic activity (51), and in energy and nitrogen metabolism as a whole (52, 53). It is from such studies that we may expect a comprehensive understanding of all the influences exerted by a growing tumour upon the tissues of its host—whether general or particular, specific or non-specific, essential or not.

Summary. 1. Growth of the Walker rat carcinoma 256 (and of

continued, with the addition for groups III and IV of aneurin 1 mg. riboflavin 2 mg, nicotinamide 20 mg, pyridoxin 2 mg, calcium pantothenate 3 mg, and folic acid 1 mg, for each batch daily. The weights of the carcases, seminal vesicles and tumours (at 2 weeks), are shown in Table 9, from which it is clear that although atrophy

Table 9

The influence of high protein diet supplemented with the vitamin B complex

Stock Diet					Breeding diet and supplement				
Group I		Group II			Group III		Group IV		
C (g)	SV (g)	C (g)	WT (g)	SV (g)	C (g)	SV (g)	C (g)	WT (g)	SV (g)
180	0.51	225	58	0.15	200	0.51	255	55	0.24
165	0.75	165	48	0.12	180	0.61	265	48	0.21
165	0.58	180	46	0.12	180	0.45	200	45	0 11
165	0.58	130	45	0 06	175	0.79	225	36	0 33
164	0.45	210	37	0.27	175	0.46	225	34	0 26
160	0 57	170	24	0.10	175	0.61	225	27	0.29
160	0.62	150	24	0.05	170	0.42	200	14	0.45
155	0 60	160	20	0.07	170	0.45	175	10	0 35
149	0.58				165	0.48	230	2	0.74
145	0.35				160	0.64			

Abbreviations: C=Carcase; SV=Seminal vesicles, WT=Walker tumour

of the seminal vesicles has not been prevented by the supplemented breeding diet, it is less pronounced than in tumour-bearing rats maintained on the stock diet alone.

Discussion

While the changes above described are of much interest in themselves, further work will be required to determine more precisely—or, rather, as precisely as is feasible, on account of their complex inter-relationships—the role of hormonal and nutritional influences in bringing them about. It is possible that the clinical state of malignant cachexia may be due to hypofunction of the adrenal, or to a pituitary-adrenal imbalance (25, 26), but in *Begg's* view (27) the thesis requires substantiation, and would not, in any event, necessarily account for all the systemic effects observed. At the moment, some preference might be given to the view that the main underlying disturbance is nutritional, imposed by the growing tumour on the tissues of the host, and leading to secondary changes in the susceptibility of many target organs to endocrine control. Such changes can without doubt be evoked by nutritional depletion, and the depleting effects of transplanted tumours in the rat have been likened by *Allison, Bernstein* and *Babson* (28)—in some

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31. *Adams, D. H.:* Brit. J. Cancer 4, 183 (1950). – 32. *Adams, D. H.:* Brit. J. Cancer 5, 409 (1951). – 33. *Day, D. D., Gabrielson, F. C.,* and *Zip-Lind, J. B.:* J. nat. Cancer Inst. 15, 239 (1954). – 34. *Greenstein, J. P.:* J. nat. Cancer Inst. 15, 1603 (19ͷͷ). ͷͷ *ͷͷͷͷͷͷͷ ͷ ͷ ͷͷͷ ͷͷͷͷͷͷ A.:* J. nat. Cancer Inst. 12, 997 (1 ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ and *Millar, J.:* Canad. J. med. ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ and *Fukuola, F.:* Jap. med. J. 1, ͷ ͷ *Fukuola, F.:* Gann 40, 45 (1949). – 39. *Nakahara, W.,* and *Fukuola, F.:* Gann 41, 47 (1950). – 40. *Fukuola, F.,* and *Nakahara, W.:* Gann 42, 55 (1951). – 41. *Nakahara, W.,* and *Fukuola, F.:* Advanc. Cancer Res. 5 (1957) (in press). – 42. *Luclé, B., Berwick, M.,* and *Zeckuer, I.:* J. nat. Cancer Inst. 13, 681 (1952). – 43. *Stewart, A. G.,* and *Begg, R. W.:* Cancer Res. 13, 556 (1953). – 44. *Stewart, A. G.,* and *Begg, R. W.:* Cancer Res. 13, 560 (1953). – 45. *Boyd, E. M., Connell, M. L.,* and *McEwen, H. D.:* Canad. J. med. Sci. 80, 471 (1952). – 46. *Boyd, E. M., McEwen, H. D.,* and *Shanas, M. N.:* Canad. J. med. Sci. 31, 493 (1953). – 47. *Boyd, E. M., Boyd, C. E., Hill, J. G.,* and *Ravinsky, E.:* Canad. J. Biochem. Physiol. 32, 359 (1954). – 48. *Boyd, E. M., Fontaine, V.,* and *Hill, J. G.:* Canad. J. Biochem. Physiol. 33, 69 (1955). – 49. *Boyd, E. M.,* and *Tilkala, A. O.:* Canad. J. Biochem. Physiol. 34, 259 (1956). – 50. *Boyd, E. M., Kelly, E. M., Murdoch, M. E.,* and *Boyd, C. E.:* Cancer Res. 16, 535 (1956). – ͷͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ *Fͷͷ ͷnger, L. P.,* and *Mider, ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ r, G. B.:* Some tumor-ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ lian Cancer Research ͷ ͷ ͷ ͷ ͷ ͷ ͷ ͷ w York 1955.

certain other tumours both transplanted and induced) is accompanied by progressive atrophy of the seminal vesicles, and, to a somewhat less extent, of the prostate.

2. While little or no alteration takes place in the weight of the testes, the Sertoli cells show an increase in their number and in their content of lipid.

3. Marked hypertrophic changes occur in the adrenal—similar to those already noted by others—while the anterior lobe of the pituitary may exhibit, although not constantly, an increase in size and in the number of chromophobe cells.

4. The atrophic changes in the accessory organs are reversed or annulled by treatment with testosterone propionate or with serum gonadotrophin, and by implantation of fresh pituitary glands and are less pronounced in animals maintained on a high protein diet supplemented with the vitamin B complex.

1. See 28th Annual Report of the British Empire Cancer Campaign 1950, 76; 29th Annual Report of the British Empire Cancer Campaign 1951, 32. – 2. *Haddow, A., Kon, G. A. R.,* and *Ross, W. C. J.:* Nature 162,

C. R.: J. Urol. (Baltimore) 47, 31 (1942). – 7. *Mann, T.:* The Biochemistry of

1C

11. *Süre, B., Theis, R. M.,* and *Harrelson, R. T.,* with the assistance of *Farber, L.:* Amer. J. Cancer 86, 252 (1939). – 12. *Ball, H. A.,* and *Samuels,*

Kropp, B. N., and
14. *Moore, C. R.,*
(1930). – 15. *Ham,*
Porter, J. C., and
Melampy, R. M.,
C. K., and *Men-*
delsohn, O.: Growth 20, 53 (1956). – 19. *Greenberg, S. M.,* and *Ershoff, B. H.:* Proc. Soc. exp. Biol. (N.Y.) 78, 552 (1951). – 20. *Moore, C. R.,* and *Price, D.:* Amer. J. Anat. 50, 13 (1932). – 21. *Moore, C. R.,* and *Samuels, L. T.:* Amer. J. Physiol. 96, 278 (1931). – 22. *Lutwak-Mann, C.,* and *Mann, T.:* Nature 165, 556 (1950). – 23. *Lutwak-Mann, C.,* and *Mann, T.:* Biochem. J. , 62 (1949). – 24. *Gold-smith, E. D.,*
25. *Begg, R. – 26. Dalton, A. J.:* J. nat Cancer Res. 11, 341 (195 *Babson, A.:* Fed. Proc. 1 Soc. Med. 47, 27 (1954). – 30. *Greenstein, J. P.:* J. Amer. med. Ass. 148, 697 (1952). –

found in the control animals did not differ from those
~~seen in the~~ ... (illegible) ...

two sarcomata and four carcinomata of the lungs.
When the results were collected it was clear that the
administration of maleic hydrazide by mouth or by

to 0·001 M, maleic hydrazide had no effect on mitosis
or cell division in the mouse epidermis.

Maleic hydrazide was also tested upon skin from
the guinea pig's ear grown in tissue culture in a
medium containing serum, glucose, Krebs-Ringer
phosphate and streptomycin hydrochloride. Up to a
concentration of 0·01 M, maleic hydrazide had no

Table 1. TEST FOR CARCINOGENIC PROPERTIES OF MALEIC HYDRAZIDE ON RATS AND MICE

Species Route of administration Sex	Rats								Mice							
	Injection 500 mgm /kgm./week				Feeding 1 per cent maleic hydrazide				Injection 500 mgm /kgm./week				Feeding 1 per cent maleic hydrazide			
	Male		Female		Male		Female		Male		Female		Male		Female	
Group C = Control T = Treated	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
Numbers:																
1. Start	10	14	10	15	10	15	10	15	10	15	10	12	10	15	10	15
2. Alive at 52 weeks	8	12	10	15	8	13	8	14	10	15	10	12	10	15	9	15
3. Alive at 100 weeks	4	2	2	5	3	4	4	5	0	0	0	3	3	4	3	4
4. Histological examination	9	11	9	15	8	14	9	14	3	7	7	7	3	9	4	8
5. Autopsy only*	2	6	0	1	3	6	0	1	1	8	3	5		4		8
Average survival†(weeks)	84	72	88	72	85	67	61	76	83	85	81	83	94	89	74	90
Range	33-93	7-90	61-99	52-93	17-98	23-96	8-83	38-96	72-91	57-89	73-93	71-89	77-93	79-99	19-97	77-88
Gain in weight‡(gm. in first 52 weeks)	406	360	206	228	272	263	177	163								
Tumours																
Malignant	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	1
Lymphoma	2	0	0	1	1	0	0	0	0	0	0	2	0	0	0	1
Benign	0	0	3	5	0	1	2	3	1	4	6	2	1	2	0	5
Total	2	0	4	7	1	2	2	4	1	4	6	5	1	2	0	7

* In many mice autolysis was too far advanced to make histological examination worth while.
† The survival time of those animals that died in less than 100 weeks
‡ Rats receiving the diet as a powder grow less rapidly and do not reach the final weight of those receiving the diet as cubes.

weekly injection to rats and mice over a period of
two years had not resulted in any increase in the
incidence of tumours (Table 2). In this experiment,
twenty-nine rats received weekly subcutaneous
injections of maleic hydrazide, but only one of the
animals developed a sarcoma at the injection site.
This contrasts with the figure of three out of the
total of fifty-two noted in the preliminary experi-
ment which was carried out on rats of the same stock.

The possibility that any carcinogenic property
possessed by maleic hydrazide might be enhanced by
the administration of a co-carcinogen was tested by
applying maleic hydrazide with croton oil to mouse
skin. Two mice developed tumours out of twenty-
one treated, but seven mice out of fifty-three devel-
oped tumours with croton oil treatment alone[9].
This finding indicated that maleic hydrazide had no
tumour-initiating action on mouse skin (for definition
see Salaman and Roe, Brit. J. Cancer, 7, 472 : 1953).
Further tests for carcinogenicity by prolonged
application alone to mouse skin were not carried out,
because so far it has been found that all substances
carcinogenic for mouse skin are also initiators of
carcinogenesis in the same tissue. (However, not all
initiators of carcinogenesis are also carcinogenic in
mouse skin, though they may be for other tissues.)

In a further experiment, pellets of cholesterol con-
taining 20 per cent maleic hydrazide were implanted
into the bladders of mice. No tumours developed
in a group of fourteen mice which survived forty
weeks[4].

Further work was done in order to see whether
maleic hydrazide displayed any action on growing
mammalian cells. Large doses (125 mgm /kgm.) were
given to rats bearing Walker tumours, but no effect
on tumour growth or cell mitosis was observed. The
effect on mammalian cells was further examined by
studying the development of mitosis in fragments of
mouse ear epidermis grown in a phosphate buffer
containing glucose. In concentrations from 0·0001

gross or microscopical effects upon the explants. The
same concentration of maleic hydrazide was also
without effect upon the respiration of skin cells
studied 2 and 22 hr. after adding the maleic hydrazide.

An attempt to follow the metabolism of maleic
hydrazide in mammals was made in a study on

Table 2. SUMMARY OF TUMOURS FOUND IN RATS AND MICE RECEIVING MALEIC HYDRAZIDE

Tumour	Rats Control	Rats Treated	Mice Control	Mice Treated
1. Fibroepithelial mammary tumour	5	7	—	—
2. Lymphoma	3	1	4	3
3. Adenoma of lung	—	—	4	10
4. Carcinoma of lung	1	2	—	1
5. Hepatoma	—	—	1	2
6. Fibroma	—	1	—	—
7. Sarcoma	—	1	—	—
8. Pituitary adenoma	—	1	—	—
Total incidence	9/40	13/59	10/40	16/57

rabbits. After an oral dose of 100 mgm./kgm.
... (illegible) ...
metabolites such as glucuronides or ethereal sulphates
were found, so that the 40 per cent maleic hydrazide
unaccounted for may either be excreted slowly over
long periods or else is broken down and destroyed.

In conclusion, it may be stated with reasonable
... without significant
... animals or isolated
... ng evidence against
... vity comparable to
The absence of any

THE NON-TOXICITY OF MALEIC HYDRAZIDE FOR MAMMALIAN TISSUES

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IN 1949 maleic hydrazide (I), 1 : 2 dihydropyridazine-3 : 6 dione, was introduced as a plant growth regulator and has since found a number of important commercial uses as a selective weed killer and as a depressant of plant growth in a variety of circumstances. The mode of action of maleic hydrazide on the plant cell is not clearly understood. There is some evidence that it may interfere with the metabolism or action of the natural growth regulators of plant cells (auxins)[1,2].

By the conventional tests used to study mammalian toxicity, maleic hydrazide appears to be virtually harmless, and large doses are tolerated by rats, mice, guinea pigs, dogs and hens. The LD50 for rats is stated to be 4 gm./kgm. and rats were unaffected by a life-time on a diet which contained up to 1 per cent maleic hydrazide[3]. In confirmation of this, we observed in preliminary experiments no effect on the growth of young rats receiving a diet containing 1 per cent maleic hydrazide. The fact that the growth of plant cells may be affected by concentrations of 0·001 per cent suggests that the action of maleic hydrazide is in some way specific for plant, as distinct from mammalian, tissues. However, during the study of the action of maleic hydrazide on *Vicia faba* roots, Darlington and

McLeish[4] found that the arrest of cell division was accompanied by chromosome breakage. They noted that maleic hydrazide causes breakage of the heterochromatin of the plant cells, whereas X-rays break only the euchromatin. Nevertheless, they concluded as follows: "Since nearly all chromosome breaking agents have so far proved to be cancer producing as well, we must hope that the agricultural use of this new agent will not be encouraged before suitable tests are made".

What follows is a summary of the work that has been done since the above communication appeared, in an attempt to show whether or not maleic hydrazide possesses any carcinogenic properties for mammalian tissues. In the meanwhile, the warning had been taken very seriously by makers and users of maleic hydrazide in Britain, and the use of maleic hydrazide has been restricted to situations where contamination of food crops would not occur. Nevertheless, there are potential uses for maleic hydrazide in suppressing sprouting of stored vegetables, for example, where food contamination might take place. Apart from the specific problem as it relates to maleic hydrazide, this account illustrates some of the difficulties of accumulating satisfactory evidence in order to substantiate the negative assertion that a given substance is not a carcinogen.

Soon after Darlington and McLeish's communication appeared, experiments were started at the Chester Beatty Research Institute in which solutions of maleic hydrazide (1 c.c. of the diethanolamine salt, each c.c. containing 5 mgm.) were injected subcutaneously at approximately weekly intervals for fourteen months into rats. Of the total of fifty-two rats so treated in two experiments, three developed sarcomata, whereas of the controls receiving saline none developed tumours. Although rats develop sarcoma after the injection of foreign materials, the incidence in this particular experiment was unusually high and a further experiment was planned using rats and mice. The results from this experiment are summarized in Table 1. Groups of young males and females of each species were given maleic hydrazide either by a weekly subcutaneous injection of a dose of 500 mgm./kgm. (monosodium salt) in aqueous solution or received a standard diet (MRC 41 in powder form) containing 1 per cent maleic hydrazide added as a dry powder. The experiment ran for 100 weeks. Control animals received weekly injections of the same volume of saline or the same powdered diet without the addition of maleic hydrazide. Unless post-mortem autolysis was advanced, macroscopical and microscopical examinations of all the dead animals were carried out. As the majority of the animals were aged at the time of their death, they showed a wide range of pathological changes in their organs, particularly in the lungs and kidneys.

Clearly maleic hydrazide had no effect on the growth and general health of the rats or mice. A special search was made for tumours and many doubtful lesions were subjected to separate and independent scrutiny by two of us (R. D. P. and P. N. M.). The most common tumours were the fibroepithelial mammary tumours in the female rats and lung adenomata in mice. These tumours were of the types commonly encountered in the ordinary laboratory strains of rats and mice. The possible malignancy of some of these tumours may be a matter of debate, but in this experiment the types

Cholesterol as a carcinogen

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[Plate 2]

In confirmation of previous reports from this Institute, cholesterol has been shown to be carcinogenic. Highly purified cholesterol (Schwenk process), dissolved in olive oil by heating on the water-bath, was injected subcutaneously into 224 stock mice. Fourteen sarcomas were induced at the site of injection. In one group of these mice, eleven sarcomas arose in 115 mice, the percentage when calculated on the number of mice which survived for at least the minimal latent period being 14 %. There is no evidence that purification impairs the carcinogenic potency of cholesterol.

INTRODUCTION

The evidence that purified cholesterol is a carcinogen was put forward in earlier reports from this Institute (Hieger 1947, 1949; Hieger & Orr 1954).

Further experiments have now been completed which reinforce the statements presented there. Cholesterol purified by the bromination technique of Schwenk (1952) was found to give a product which, after one further crystallization from acetone, had no absorption in ultra-violet light above about 230 to 237 mμ and indeed one batch had no absorption above 225 mμ. The purification process used for the earlier preparations (described in Hieger & Orr 1954) consisted of six stages, viz. acetylation, bromination, de-bromination, chromatography, de-acetylation and crystallization. The u v. absorption curve of the resulting purified cholesterol fell rapidly in the 225 mμ region and flattened out between about 230 and 260 mμ.

METHOD

If the u.v. absorption curve is accepted as the principal criterion of the purity of the cholesterol, then it would appear that the simpler purification process of Schwenk gives a purer end-product. In explanation, it is possible that as the purification processes are multiplied, traces of undesirable substances are introduced increasingly into the material. For the detailed description of the injection technique, the characteristics (histology and transplantation) of the tumours induced, and of the results of control tests on solvents in a large series of mice (700 animals, see below), see the previous paper in this series (Hieger & Orr 1954).

The cholesterol purified by the Schwenk process (which will now be referred to simply as 'Schwenk cholesterol') has been tested on for carcinogenic activity upon 224 mice. These were divided into two groups: (a) a series housed in the 'hydrocarbon-carcinogen-free room' (where no such compounds were in use), and (b) a series kept in the 'carcinogen room' where some other experiments were being carried out which involved the use of potent carcinogens, chiefly 3:4-benzpyrene.

[84]

significantly greater number of tumours in male or female rats and mice receiving maleic hydrazide by two routes over a period of two years makes it improbable that it is even a weak carcinogen for these species or that some metabolite of maleic hydrazide has any such effect. A further study of the mode of action of maleic hydrazide on plant cells may perhaps eventually help us to understand why the chromosomes of plant cells but not those of mammalian cells are broken when cells are acted on by maleic hydrazide. Meanwhile, it seems reasonable to claim that proper tests have now been made, and the weight of evidence provided by the results of these tests is that maleic hydrazide has no carcinogenic properties.

[1] Leopold, A. C., and Klein, W. H., *Science*, 114, 9 (1951)
[2] Waggoner, P. E., and Dimond, A. E., *Science*, 117, 13 (1953)
[3] Information from Naugatuck Chemical International, New York, U.S.A.
[4] Darlington, C. D., and McLeish, J., *Nature*, 167, 407 (1951)
[5] Salaman, M. H., and Roe, F. J. C., *Brit. J. Cancer*, 10, 363 (1956)
[6] Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S., and Watson, J. G., *Brit. J. Cancer* (in the press)
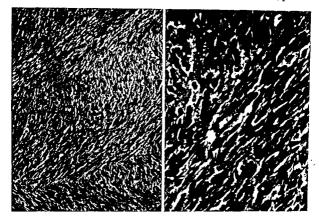
FIGURE 1. Sarcoma at site of injection induced in a ♀ stock mouse by olive-oil solution of purified cholesterol. 16th month. (Magn. × 80.)
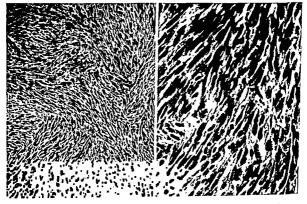
FIGURE 2. Same tumour as figure 1. (Magn. × 355.)

FIGURE 3. Another sarcoma induced by same technique as in figure 1. ♂ stock mouse. 18th month. (Magn. × 80.)

FIGURE 4. Same tumour as figure 3. (Magn. × 355.)

RESULTS

Unfortunately, an epidemic broke out in the non-carcinogen room and some hundreds of mice were lost, including those injected with Schwenk cholesterol, but not before sarcomas had developed in three of them, and had been proved histologically (see figures 1 to 4, plate 2). The latent periods of these three (8½, 9 and 10 months) were unusually short for cholesterol-induced tumours. The epidemic did not spread to the 'carcinogen room' where the series of mice injected with Schwenk cholesterol had a reasonably high survival rate (although not as high as in some other series in this room). Altogether eleven sarcomas were obtained in this series, the first three of which appeared after short latent periods (9½, 11 and 11 months).

TABLE 1. SARCOMA INDUCTION IN STOCK MICE BY HIGHLY PURIFIED
CHOLESTEROL (SCHWENK PROCESS) IN OLIVE OIL

		survival rate (months)									no. of sar-comas	latent period (months)	% incidence of sarcomas per 100 mice calculated on no. of mice	
expt.	location	at start	6	9	12	15	18	21	24	27			at start	at minimal latent period
1	'non-carci-nogen room'	109	107	102	(all mice killed: epidemic)						3	8½, 9, 10	—	—
2	'carcinogen room'*	115	90	84	66	33	22	10	—	—	11	9½, 11, 11, 16, 17, 17, 18, 20, 22, 22, 23	9 5	14
For comparison: a test† on stock mice in the 'carcinogen room' using commercial cholesterol in olive oil														
3	'carcinogen room'	100	—	—	75	64	45	32	10	2	8	11, 12, 15, 16, 17, 20, 20, 24	8	19 7

* For a detailed description of this term see Hieger & Orr (1954).
† The figures for this experiment have been reported previously (Hieger & Orr 1954).

The close similarity of the latent periods of the two sets of three sarcomas (i.e. the series in the carcinogen room and in the non-carcinogen room) leaves no doubt at all that had the epidemic been avoided, the subsequent history of tumour development in the non-carcinogen room in mice injected with Schwenk cholesterol would have resembled closely that of the parallel series in the carcinogen room. The data in table 1 show the yield of sarcomas and the survival rate for the two series, and for comparison the corresponding figures for a test of unpurified commercial cholesterol.

The similarity of the results of experiments 2 and 3 (table 1) shows that the solutions of commercial and of highly purified cholesterol have much the same carcinogenic potency, and suggests strongly that cholesterol *per se* is the carcinogen in commercial cholesterol, for here the kind of solvent, strain of mice and environment were kept constant in these two experiments. Alternatively, if the immediate carcinogen is produced *in vivo* from cholesterol, it is cholesterol which is the parent compound and not some associated substance present as impurity in commercial cholesterol.

resistance of certain groups of mice to the carcinogenic stimulus of cholesterol, and for the pronounced response of other groups. The foregoing results support the following conclusions:

(1) Approximately 10% of mice (Stock or C_{57}) surviving a minimum of one year, ultimately develop sarcoma at the site of injection when injected subcutaneously with a solution of cholesterol in olive oil prepared by heating on the water-bath.

(2) Purification of cholesterol (as judged by u.v. absorption) does not impair its carcinogenic activity.

(3) Housing mice in the same room with others which are being treated with 3:4-benzpyrene does not introduce a serious risk of contamination.

TESTS ON COMPOUNDS RELATED TO CHOLESTEROL

In the earlier stages of this investigation, before solutions of purified cholesterol had been shown to have carcinogenic activity, and even before commercial cholesterol had been tested, sarcoma induction by the unsaponifiable fraction of human organs (liver, lung, kidney, muscle) suggested experiments carried out between 1941 and 1946, to test some members of the steroid group of compounds. Later, when such tests had given negative results, one was led to infer that cholesterol *per se* might be the essential carcinogen in the unsaponifiable fraction.

In table 3 are shown the results of the tests on compounds related to cholesterol. None of the steroids tested showed appreciable activity as carcinogens, but of course the number tested represents only a small fraction of all those which are now available. With regard to experiments 10 and 11 (deoxycholic acid and sodium deoxycholate), the single sarcomas obtained in each test show that the activity of these preparations is of the same order as that of the solvents. Fieser, Bischoff and co-workers (1946, 1954, 1955, 1955) have prepared a number of oxidized derivatives of cholesterol which are being tested.

THE ROLE OF SOLVENT

Only oily solutions of cholesterol have shown carcinogenic activity; no tumours were obtained in an experiment where a suspension of cholesterol in a 4% gelatin gel was used. As far as these tests go it would appear that the oil is a necessary part of the injection material. The control tests totalled about 700 mice treated with olive oil or lard which had been heated on the boiling water-bath; two sarcomas were obtained with these oils. Similar low orders of activity have been reported by Bischoff (1955), by Steiner (1943, 1947) and by other workers. Burrows, Hieger & Kennaway (1936) found that lard gave seven sarcomas and olive oil one sarcoma in 198 rats, but no tumours in 236 mice. Hartwell's bibliography (1951) of compounds which have been tested for carcinogenic activity describes some hundreds of tests with the female sex hormones. The great majority of these were carried out with olive oil or sesame oil as solvents, yet in only one or two cases did the hormones dissolved in oil induce sarcoma. The above facts show that olive

The high incidence of sarcomas in experiments 2 and 3 in table 1—high, that is, for this type of carcinogen (cholesterol-rich preparations have in some of our previous experiments shown a much lower production of sarcomas)—cannot be easily explained away on the grounds of contamination by 3:4-benzpyrene conveyed from other mice in the same room ('carcinogen room') which were being treated with these compounds, for the following reasons:

TABLE 2. SARCOMA INDUCTION IN MICE BY INJECTION OF COMMERCIAL CHOLESTEROL IN OLIVE OIL

| expt. | mouse strain | location | no. of mice at start | no. of mice surviving (months) | | | | | | | | | no. of sarcomas | sarcoma induction calculated per 100 mice | | latent period (months) |
				6	9	12	15	18	21	24	27	30		at start (%)	at 1 yr. (%)	
1*	C_{57}	non-carci-nogen room	60	34	30	28	21	14	9	3	1	—	1	1·7	3·6	22
2	C_{57}	non-carci-nogen room	30	10	10	10	9	7	7	5 (3 at 25 m.) epidemic (all killed)			1	3·3	10	23
3	C_{57}	non-carci-nogen room	39	28	26	24	20	16	11†	epidemic (all killed)			3	7·7	13	12, 14, 18
4	C_{57}	carcinogen room	75	62	48	46	40	26	15	3	0	—	4	5·5	8·7	21, 22, 22, 23

* This experiment was reported (Experiment A, Table V) by Hieger & Orr (1954).
† Eleven mice were still alive in the 21st month of this experiment (no. 3) There can hardly be any doubt that but for the epidemic the percentage yield of sarcomas would have been still higher.

Hieger & Orr (1954) stated that the percentage sarcoma induction (stock mice) was 8% in the carcinogen room (CR) and 4·5% in the non-carcinogen room (non-CR). If the calculation is made to exclude the mice which did not survive 1 year and were therefore not at risk, the percentages then rise to 10·7% for the CR and 7·9 and 5·4% (two experiments; mean 6·7%) for the non-CR. Taking into account the difference between the two percentage yields of tumours, i.e. 10·7 and 6·7%, and the total number of mice involved in the experiment, i.e. 235, comprising 100 in CR and 70 and 65 (two experiments) in non-CR, this difference could not be considered a very serious divergence; at any rate a difference of such magnitude might well be incurred by variations in the 'sensitiveness' of the mice, a factor about which we know nothing as yet. For example, several experiments have shown that sarcoma development by cholesterol is more frequent in the fitter mice than in those groups which are obviously less healthy. Four experiments are detailed in table 2 to show the differences in sarcoma yield obtained when conditions are very similar, i.e. when the same material is injected in four separate groups of mice of the same strain. This table illustrates the order of similarities and differences which can be expected when a test on the carcinogenic potency of cholesterol is repeated a number of times.

The author is inclined to the explanation that some unidentified factor linked with the health of the mice, or some minor genetic factor, would account for the

Fieser, L. F., Greene, T. W., Bischoff, F., Lopez, C. & Rupp, J. J. 1955 *J. Amer. Chem. Soc.* 77, 3928.

Hartwell, J. L. 1951 *Survey of compounds which have been tested for carcinogenic activity.* Bethesda, Md.: National Cancer Institute.

Hieger, I. 1947 *Nature, Lond.,* 160, 270.

Hieger, I. 1949 *Brit. J. Cancer,* 3, 123.

Hieger, I. & Orr, S. F. D. 1954 *Brit. J. Cancer,* 8, 274.

Schwenk, E. & Wertessen, N. T. 1952 *Arch. Biochem. Biophys.* 40, 334.

Steiner, P. E. 1943 *Cancer Res.* 3, 385.

Steiner, P. E., Stanger, W. D. & Bolyard, H. N. 1947 *Cancer Res.* 7, 273.

oil, lard and sesame oil* have very low potency as carcinogens; less than 1% of injected mice develop sarcoma. Most probably the oil is necessary in the process of carcinogenesis by cholesterol to ensure solution.

TABLE 3. TESTS FOR CARCINOGENIC ACTIVITY OF COMPOUNDS RELATED TO CHOLESTEROL. INJECTIONS WERE MADE SUBCUTANEOUSLY, REPEATEDLY

| expt. | compound | how administered | no. of mice at start, and surviving during expt. | | | | | | | no. of sarcomas |
			at start	after 1 yr	18m.	24m.	27m.	30m	33 m	
1	isocholesterol	15% solution in lard	10	9	5	4	0	—	—	0
2	isolumisterol	15% solution in lard	10	9	4	0	—	—	—	0
3	cholesterylene	(15%) saturated solution in lard	10	5	5	3	2	1	—	0
	cholesterylene not purified	10% solution in lard	10	9	7	2	1	0	—	0
4	ergosterol	15% solution in lard	10	10	3	0	—	—	—	0
5	Δ⁴-cholestene	15% solution in lard	10	9	4	3	2	0	—	0
6	7-dehydrocholesterol	15% solution in lard	10	10	7	2	2	0	—	0
	7-dehydrocholesterol	15% solution in lard	10	9	4	0	—	—	—	0
7	epicholesterol + allocholesterol	15% solution in lard	10	9	8	3	3	2	0	0
8	7-hydroxycholesterol (mixture of 7 (α) and 7 (β))	15% solution in lard	20	14	6	2	1	1	1	0
9	lathosterol	10% solution in olive oil	50	37	23	5	2	0	—	0
10	deoxycholic acid	1% suspension in olive oil + tristearin (5:1)	73	34	21	6	1	0	—	1*
11	Na-deoxycholate	1·2 g cholesterol + 60 mg deoxycholate + 12 g olive oil + tristearin	62	44	26	6	0	—	—	1†

* C₈₇ mouse ♂, latent period; 21 months.
† C₅₇ mouse ♂, latent period; 18 months.

REFERENCES

Bischoff, F. & Rupp, J. J. 1946 *Cancer Res.* 6, 403
Bischoff, F., Lopez, C. & Rupp, J. J. 1954 *Abstr. Amer. Chem. Soc.* March 3-C.
Bischoff, F., Lopez, C., Rupp, J. J. & Gray, C. L. 1955 *Fed. Proc.* 14, 183.
Burrows, H., Hieger, I & Kennaway, E. L. 1936 *J. Path. Bact.* 43, 419

* Such oils are liable to chemical changes on keeping, especially if they have been heated and exposed to atmospheric oxidation. These changes are accompanied by an increase in absorption in the u v in the region 220 to 250 mμ. There is no evidence, however, that any such change which might develop after the cholesterol is heated on the water bath with the only solvent contributes to the carcinogenic activity of the resulting solution

neoplastic growths, as we know, arise in organs of the endocrine system or else in organs under its direct influence. They are composed of cells which have been stimulated to proliferate because of a hormonal imbalance, and in many instances may be fully or partially controlled by a restoration of the normal equilibrium brought about by a readjustment of the endocrine balance.

Autonomous neoplasms on the other hand, such as those which develop in the skin, stomach, lung, etc., are not under direct endocrine control nor can their growth or behaviour be influenced by any form of hormonal modification. However, as in all biological problems, the dividing line between these two basically different types of cancer is not as rigid as might at first appear, because a certain number of hormone-dependent tumours must obviously give rise to autonomous variants.

When a tumour becomes an autonomous lesion it is composed of permanently altered cells and is freed from its sensitivity to the hormonal forces which control the cells of the endocrine-dependent tumours. It is of course conceivable that some malignant growths might be composed of both the dependent and autonomous cellular components, and this might explain why a hormonal-dependent tumour whose growth has previously been inhibited by means of endocrine therapy, will suddenly lose its responsiveness to a particular hormone, and become an uncontrolled autonomous neoplasm.

In order to appreciate the first endocrinological approach to the cancer problem, we shall have to retrace our steps back to May 1932 when Lacassagne, of the Radium Institute in Paris, published his classical discovery that the naturally occurring oestrogenic steroid hormone 'Oestrone' was implicated in the cause of breast cancer in mice. This was the first demonstration that a hormone circulating in the blood-stream is capable of inducing neoplasia. This experiment of Lacassagne (1932) was in fact the foundation-stone in the pathway of endocrine carcinogenesis. Since then, great strides have been made in the induction, inhibition and prevention of certain forms of endocrine cancer in both man and animals by altering their hormonal environment.

Some Anomalies in
Endocrine Carcinogenesis

E. S. HORNING

IT is actually inside a generation since the chemist and the biologist have made important discoveries on the causes of cancer, which have been responsible for directing researches into two main channels. There are the extraneous agents such as the carcinogenic hydrocarbons and the ionizing radiations and the inherent agents such as the hormones, then of course there are the genetic factors which influence responsiveness to both these extrinsic and intrinsic agents.

I propose to deal with the induction, control and prevention of endocrine neoplasia, and I intend to discuss some of the anomalies which I have personally encountered in endocrine cancer, because very often by studying the exceptions to the general rule we develop a better understanding of such elusive biological mechanisms.

I should like to begin by quoting some opening remarks made by Dr. Jacob Furth (1955) of the Cancer Research Foundation, Boston, at the recent Laurentian Conference on Hormone Research and Abnormal Growth. He made the following statement: 'Recent studies have strengthened our concept that there are two basically different types of cancer, one dependent and the other autonomous.'

The terms 'hormone-dependent' and 'hormone-independent' cancers were first used by Huggins of Chicago to denote those neoplasms which depend upon hormones for their maintenance and those which grow independently of them. Dependent

CARCINOMA OF THE PROSTATE GLAND

There is no doubt that an endocrine dysfunction is an etiological factor in the cause of prostatic cancer in man, for it has been shown that tumours of the prostate can arise by an alteration of the hormonal environment.

In man the growth of most tumours of the prostate can be held in check for considerable periods by the adoption of anti-androgenic measures, as Huggins and Hodges (1941) have admirably shown. The induction, however, of prostatic tumours in rodents and even in many higher animals constitutes an anomaly, for so far there have been no successful attempts to produce malignant prostatic cancer in these animals by the administration of hormonal agents. A glandular carcinoma of the prostate, however, was produced by Horning (1946), not by hormonal administration but by treatment with one of the carcinogenic hydrocarbons, namely 20-methyl-cholanthrene. Slices of mouse prostate, which had been carefully wrapped around crystals of this compound, were implanted subcutaneously into host mice of the same strain. Some of the prostatic tumours which subsequently developed were glandular-cell carcinomas, but most were of the squamous-cell variety. It was found that the glandular cancers could all be successfully grafted into intact mice, but could not all be successfully grown in host mice castrated before puberty. Some of these tumours would grow in castrated mice providing the host animals were treated with testosterone propionate. Later it was found that those tumours which were dependent upon the male sex hormone for their sustained growth as grafts were glandular-cell carcinomas, whereas the prostatic tumours which had become hormone-independent and were growing as autonomous lesions had recently undergone squamous metaplasia during serial transplantation. The glandular-cell tumours were hormone-sensitive, but once they had undergone squamous metaplasia they became androgen independent. These experiments are of interest for two reasons. First, because it was found possible to induce a hormone-dependent tumour in a gland under endocrine control with a carcinogenic hydrocarbon, without the direct intervention of a hormonal agent. Secondly,

Lacassagne in 1937, Nathanson and Andervont (1939), Cramer and Horning (1938) working independently were among the first to demonstrate that the spontaneous development of mammary cancer in some strains of mice could be prevented, and the incidence of cancer in other strains considerably reduced, by early treatment with a hormone antagonist.
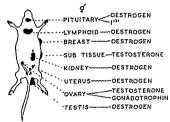

FIG. 1. Endocrine carcinogenesis. Diagram showing site of neoplasm together with responsible hormonal agent.

In the clinical field Loeser (1940) was the first to use testosterone in the treatment of breast cancer. In 1944 Haddow and his co-workers, relying on the depression of pituitary function and hence the inhibition of ovarian secretion in preference to employing a hormone antagonist, were the first to use oestrogen for the treatment of breast cancer in women. This treatment was confirmed by Nathanson (1947) who found it to be effective in post-menopausal women suffering from this disease. Pearson (1955) in a recent review claims remission in 45 per cent of cases.

Let us now investigate some of the experimental evidence upon which some of this work we have briefly discussed is based. It is proposed to discuss the induction of tumours of the prostate, kidney, pituitary, testes and ovary (see Figure 1), and also in some instances their prevention by using an appropriate hormone antagonist. These tumours have been purposely selected because in some instances both their induction and behaviour are anomalous.

neoplasia in the male but not in the intact female hamster. This work has been confirmed and extended by Horning and Whittick (1954). The several interesting problems which this investigation has brought to light are not yet fully appreciated. For instance, neoplasms in animals other than the hamster which are induced by hormones and are dependent upon them for their sustained growth, generally arise in organs of the body which either belong to the endocrine system or else come under the influence of the anterior pituitary gland. Hamsters are the exception to this rule in that the kidney, except as a part of the body subject to the general stimulation of somatotrophin, is not directly influenced by the pars anterior.

These oestrogen-induced kidney tumours possess all the histological characteristics of malignant lesions. They arise in the cortex in association with either the distal or proximal tubules, and later invade the medulla and the renal pelvis. They also metastasise. Secondaries are seen in the body cavity as well as in the lung and liver.

Another anomaly is that prolonged treatment with oestrogens invariably produces pituitary tumours of the pars intermedia, whereas in other rodents similar treatment induces chromophobe lesions of the anterior lobe.

ABSORPTION RATES OF IMPLANTED PELLETS

The differences in absorption rate between stilboestrol pellets implanted subcutaneously in the hamster, desert rat and albino rat are illustrated in Figure 3. The absorption rate of the hamster is slower than in the other two rodents, and this might explain why hamsters are able to tolerate such large amounts of stilboestrol.

It will be seen in Figure 3 that a deflection in the absorption rate of the tablets in each of the three rodents occurs on approximately the fourteenth day after subcutaneous implantation, at a period when the pellets become encapsulated. Cowie and Folley (1945) contend that this falling off is due solely to a sudden decrease in the surface area of the pellets.

because once the chemical constitution of the prostatic cancer cell had altered, it ceased to be dependent on the male sex hormone for its sustained growth. This is in fact, to use the terminology of Furth, an example of an autonomous variant.

Fig. 2. Silhouette chart showing dependence of transplantable prostatic mouse carcinoma on androgens.

Here is an endocrine-dependent tumour becoming independent of hormonal modification once it had undergone a spontaneous cellular differentiation (see Figure 2). Special emphasis has been laid on this mouse prostate work not merely because it constitutes an anomaly in endocrine carcinogenesis, but also because this work has recently been confirmed and extended by Dr. Mirand (1955) of the Memorial Institute at Buffalo.

RENAL NEOPLASIA INDUCED BY OESTROGENS

Let us now pass on to the induction of kidney tumours by oestrogen administration in the male golden hamster. One of the most interesting findings in the field of endocrine carcinogenesis was made originally by Kirkman and Bacon (1949) of Stanford University. They found that prolonged treatment with naturally occurring or synthetic oestrogens induced kidney

Even although this kidney tumour has been grafted for several years and the latent period has been reduced from nearly. twelve months to four weeks, before palpable lesions develop this tumour still retains its dependency upon oestrogen for
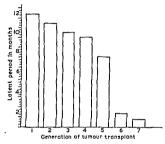

FIG. 4. Histogram showing decline in latent period
preceding growth of graft.

maintenance as a graft. If transplanted into untreated hamsters the tumour will no longer grow.

These oestrogen-induced grafted kidney tumours conform in their behaviour with many other types of hormone-dependent transplantable tumours observed in laboratory animals. Experiments were therefore undertaken, and are still in progress, to determine whether or not these primary transplantable tumours are capable of growing following abrupt withdrawal of the hormonal stimulus. Removal of the stilboestrol pellet from the subcutaneous tissues is easily achieved, and is followed by a gradual regression of the primary tumours. The regression is particularly easy to follow in grafts which have been implanted in the tail of host hamsters. The effect of this withdrawal upon the secondary and transplanted lesions is still under consideration. As these kidney tumours are hormone-dependent for their sustained growth, experiments were also undertaken to
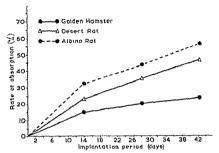
Fig. 3. Absorption of stilboestrol in golden hamster, desert rat, and albino rat.

HORMONAL FACTORS DETERMINING SUCCESSFUL TRANSPLANTATION

Many unsuccessful attempts have been made to transplant these induced primary kidney tumours into hamsters of both sexes and of varying ages. The failure of these kidney tumours to grow either as subcutaneous or intraperitoneal grafts was surprising as they possessed all the histological criteria of malignant lesions.

Consideration was then given to the fact that as the neoplasms are dependent upon high levels of oestrogen for their induction they might also be dependent upon the continued presence of this hormone in excessive amounts for their maintenance as grafts. After a long latent period the grafts grew only in oestrogen pre-treated hosts.

One of the most interesting features is the long latent period which exists between tumour transplantation and the appearance of palpable lesions. A tumour now in its eighth generation of serial transplantation is seen in the following histogram (see Figure 4). It will be noticed that the duration of the latent period between transplantation and the appearance of a palpable lesion shows a marked decline in each successive serial graft.

TABLE I. Prevention of stilboestrol-induced renal tumours in the male golden hamster with testosterone propionate

No. of hamsters	Form of treatment	Duration of treatment	Results
20♂	Stilboestrol alone (20 mg. pellets)	10½ months	18 palpable renal carcinomas 2 early cortical tumours (not palpable)
20♂	Stilboestrol + testosterone propionate (20 mg. stilboestrol) (2 5 mg. weekly in oil)	16 months	No renal tumours developed 1 hamster developed a unilateral hydronephrosis
20♂	No treatment	16 months	No spontaneous kidney tumours

INDUCED OVARIAN TUMOURS

We shall now pass on to another anomaly in endocrine cancer. This deals with the induction of ovarian tumours following regular administration of the male sex hormone commencing 48 hours after birth.

These ovarian tumours were all theca-cell lesions and were induced in albino rats eighteen months after the commencement of treatment. These tumours are of interest for two reasons; first because the ovary itself is a target organ of the endocrine system; and second, because testosterone has never been seriously regarded as a potential carcinogenic agent, although Lacassagne (1939) had claimed that repeated inoculations with the male sex hormone, under strictly controlled conditions, induce subcutaneous sarcoma.

HORMONAL IMBALANCE IN MAN

I pass now to the possible association of breast cancer in man with a spontaneous hormonal imbalance. It is established that in women the ovary, although not the sole source of oestrogen

oestrogenic hormones.

determine if tumour formation could be prevented by simultaneous treatment of the host with stilboestrol and its antagonist, testosterone propionate. If renal neoplasia could be prevented by this means, this would be additional evidence of the endocrine nature of tumours arising in a gland which is not under the direct influence of the endocrine system.

PREVENTION OF STILBOESTROL-INDUCED RENAL TUMOURS WITH TESTOSTERONE PROPIONATE

The set up of this experiment was simple. Sixty male hamsters all twelve weeks of age were divided into three separate groups, each consisting of twenty animals. The first group were treated with stilboestrol alone, the second received combined treatment with stilboestrol and testosterone propionate, and the third group were untreated.

Examination of Table 1 shows that with the exception of one, all the stilboestrol-treated hamsters developed palpable kidney tumours at various intervals up to ten to eleven months after the commencement of treatment. All these renal lesions were bilateral and multifocal and varied considerably in size. The size of the growth does not always depend upon the duration of treatment. For instance, the single hamster in this group which developed no palpable tumour at the end of this period of treatment, was found at post mortem to possess naked-eye cortical lesions in each kidney. None of the hamsters receiving the combined treatment with stilboestrol and testosterone developed any kidney tumours (see Table 1). The testes and seminal vesicles, in contrast to those treated with stilboestrol alone, showed no atrophic changes. It will also be noticed that no spontaneous kidney tumours appeared in the group of animals which received no treatment (see Table 1).

These experiments with a hormone antagonist demonstrating conclusively that kidney tumours in the hamster can be prevented by combined treatment with stilboestrol and testosterone provide additional evidence that renal neoplasia in the hamster comes under the category of hormonal cancer.

before discussing the general problems of endocrine carcino-
genesis.

The production of ovarian tumours in the albino rat following
massive doses of testosterone propionate was first observed by
the late Mr. Harold Burrows some years ago at the Chester
Beatty Research Institute. Recently I confirmed his contention.
The neoplasms were all theca-cell tumours. The histogenesis of
these lesions is obscure, but many workers regard them, and I
believe quite rightly, as endocrine lesions arising from the
stromal cells of the ovarian cortex. The results were surprising
not only because the ovary is a target organ of the endocrine
system, but also because testosterone has never seriously been
regarded as a potential carcinogen. What then is the rationale?

When I discussed these unpublished results at the last
Gordon Conference in New London, Dr. Engel (1955) said he
was not surprised since he had recently discovered that testo-
sterone was converted in the body into oestradiol. Pearson,
working at the Sloan Kettering Institute, New York, contends
that androgen sometimes accelerates the growth of mammary
cancer in women, which he attributes to the possible conversion
of androgen into oestrogen. The reason why progesterone
occasionally stimulates instead of inhibits tumour growth in
women might likewise be due to the same cause.

In view of these results, the recent work of van Eck and
Chang (1955) is of direct interest. They found that ovarian
tumours in rodents develop more readily in testosterone-treated
X-rayed mice than they do in X-rayed animals which have not
been treated with male sex hormone. When considering that
after injection androgen might possibly be converted into
oestrogen, the known capacity of the gonadal hormones to
stimulate mitoses of fibrocytes should also be taken into con-
sideration.

If this conversion does occur in vivo, then it should be possible
to induce renal neoplasia in the hamster by prolonged treatment
with the male sex hormone alone. These experiments are already
in progress.

We have also seen in the case of the hamster that continuous
administration of oestrogen can induce kidney carcinoma in an

Ever since Price in 1941 by grafting the ovaries of rats on to the ears of litter mates demonstrated that the difference in temperature stimulated the secretion of androgens instead of oestrogen, it has been shown that under certain conditions a reversal of sex hormone secretion can also occur in the testis. Bielschowsky (1954), in an ingenious experiment on induced cryptorchidism in rats, has demonstrated that the interstitial cell-stimulating hormone of the pituitary leads to a hyperplasia of the Leydig cells of the testis, which are normally associated with the secretion of androgen, but which do under these conditions elaborate oestrogens in sufficient amounts to induce proliferation of the mammary epithelium in the operated hosts. These results of Bielschowsky are of interest when examining the testes of men with breast cancer. There are indications that these breast tumours may have developed as a result of a hormonal imbalance. Frozen sections were cut off the testes after they were imbedded in gelatine. They were then stained with Sudan IV.

The cells of the adrenal cortex, the theca interna off the ovary, and the interstitial cells of the testis all contain lipids in their cytoplasm which are associated with the formation and storage of steroid hormones. A large part of the lipid is in the form of cholesterol which gives a positive reaction to the Lieberman-Burchardt test. Fat soluble dyes like the Sudan series stain the total lipid and naturally do not distinguish between specific steroids. Cytological examination of these human testes from men with breast cancer showed abnormal increase in the number of the Sertoli cells in the seminiferous tubules and a marked proliferation of the Leydig-cell tissue. Whether or not the abnormal amounts of steroid hormones elaborated by the testes in these instances initiated breast cancer and can be regarded, as Dr. Furth would say, as the intrinsic agent in the etiology of the disease, can only be regarded as a matter of speculation. It does, however, warrant further examination.

DISCUSSION

Let us briefly review some of the anomalies we have encountered in the induction of tumours of the ovary, prostate and kidney

One of the most fascinating features of endocrine tumours is their dependence upon a particular hormone, and the dramatic manner in which many regress once the hormonal stimulus is withdrawn. In the case of renal neoplasia in the male hamster we find that changes produced by oestrogen are reversible and last only as long as oestrogen is available to maintain the tumour. Another feature of interest in hormone-dependent transplantable tumours in laboratory animals is the long latent period which exists between implantation and the development of palpable lesions. As we have seen the latent period is gradually reduced during each successive serial generation of transplants. These renal tumours are now in their eighth serial generation, and are still hormone-dependent since they will not as yet grow in non-oestrogenized hosts. This is of exceptional interest, as there is a tendency for most endocrine-dependent neoplasms growing in experimental animals gradually to lose their dependence upon a particular hormone after they have been transplanted for several generations. There are only a few exceptions to this rule. Mühlbock (1954) in Amsterdam induced a rodent ovarian tumour by administration of pituitary gonado-trophins. This particular tumour is now in its fourth year of serial grafts and is still dependent upon either androgens or oestrogens for its maintenance. There are no indications that it will develop into an autonomous variant.

The question naturally arises as to whether the oestrogen acts directly on the tumour cell of the graft or whether the hormone brings about changes in the pituitary gland which in turn indirectly stimulate the latent tumour cell into its malignant phase. The long periods in which these tumour cells remain quiescent as subcutaneous grafts suggests that the pituitary might possibly be involved. In view of this, normal untreated male hamsters are now receiving transplants of pituitary tumours prior to the implantation of kidney carcinoma grafts. If these renal grafts will grow in non-oestrogenized hosts bearing pituitary tumour implants, it would give valuable information on the biological mechanism controlling the behaviour of hormone-dependency.

Although the induction of hormone-dependent prostatic

organ which is strictly not a member of the endocrine system and moreover does not come under the direct control of the anterior lobe of the pituitary gland. Furthermore, these kidney tumours are hormone-dependent lesions since the tumours regress following abrupt withdrawal of the hormonal stimulus, even although they are capable of metastasising via the lymphatic pathway. The induction of kidney tumours in the hamster is an interesting exception to the general rule, since other species of rodents after similar treatment never develop renal carcinoma. Preliminary experiments still in progress suggest that the liver of the hamster differs from that of the rat, mouse and guinea-pig, in that it is unable to cope adequately with the inactivation of oestrogens. Also other experiments have demonstrated that the renal epithelium of the hamster is endowed with a peculiar susceptibility to renal neoplasia. The results obtained with stilboestrol suggested the possibility that kidney cancer in the hamster might possibly be due partly to absorbed chemical carcinogens acting selectively on the renal epithelium during excretion. This contention was strengthened by the fact that kidney tumours could be induced in the hamster by subcutaneous injection at a remote site with 3:4 benzpyrene, while none developed in albino rats following similar treatment. It was of further interest to record that this particular carcinogenic hydrocarbon has been shown by Cook and Dodds (1933) to possess oestrogenic activity.

Another peculiarity about the hamster is the development of tumours of the pars intermedia, whereas other rodents following oestrogen treatment only develop chromophobic lesions of the anterior lobe of the pituitary. Taking into account this unique *response of the hamster pituitary to oestrogen treatment*, experiments are being undertaken to determine whether kidney tumours could develop in the absence of a functioning pituitary. Necrosis of the pituitary gland in normal untreated hamsters is produced by inserting radio-active seeds into the hypophysis via the trans-nasal route. This should determine whether the action of oestrogen in the hamster is a direct or an indirect effect. This would yield additional information on the mechanism of renal tumourigenesis in these animals.

known tumours either in animals or in man to ascertain by histological methods whether a particular tumour is hormone-dependent or not. Huggins, however, does contend that any breast cancer which has undergone anaplastic changes is invariably more resistant to hormone therapy.

Ever since Huggins and Scott in 1945 first used gonadectomy combined with bilateral adrenalectomy for controlling the growth of breast and prostatic cancer in man, many attempts have been made to find some method by which it would be possible to predict before operation whether the tumour was a hormone-dependent lesion. A promising step has been made recently by Pearson (1955) and his co-workers in New York. They found that measurements of urinary calcium excretion in men with prostatic cancer revealed the existence of two kinds of osteolytic metastases: one dependent on oestrogen and the other non-dependent. It is the former type of patient who responds to adrenalectomy which reduces oestrogen production and thus brings about a remission of tumour growth. Additional investigations may well confirm these findings and establish the value of urinary calcium determinations as a guide to the form of therapy to be taken in prostatic and breast cancer.

Recently Hadfield (1956) has also been studying methods by which it is hoped to determine beforehand whether or not patients with breast cancer possess hormone-dependent tumours. This author is of the opinion that breast tumours are more likely to regress when the production of oestrogen is diminished and the output of gonadotrophin in the urine is increased. He claims to have identified a mammatrophic factor from human urine. This factor has no oestrogenic activity, and it is not apparently found in the urine after hypophysectomy. Hadfield admits that the mammatrophic substance present in human urine is probably prolactin. It is known that the prolactin content of urine goes up during parturition and it will be necessary to distinguish between prolactin urinary output occurring normally at various times and ages from that which they claim to be indicative of breast cancer. If these tests can be made truly reliable for the clinical detection of hormone-dependent breast cancer, treatment by hypophysectomy would be established on a firmer basis.

tumours in mice by treatment with a carcinogenic hydrocarbon alone without the direct application of a hormonal agent, as we have just seen, constitutes an anomaly, some information of interest was brought to light regarding the factors regulating the behaviour of the malignant cancer cell to the male sex hormone. As long as the prostatic tumour cells remains a glandular cell carcinoma it is hormone-dependent, but as soon as it undergoes squamous differentiation it ceases to be dependent upon androgen for its sustained growth and immediately transforms into an autonomous lesion. The question therefore arises: Does the endocrine-dependent cancer cell differ in its chemical composition from the autonomous malignant cell which has lost its *responsiveness to the hormone-restraining forces? In other words* does this change from dependency to autonomy involve any fundamental change in cellular chemical patterns and metabolism? Unfortunately, these questions cannot be answered since the normal mechanism of hormone action is not yet fully understood. It is thought that hormones constitute an important set of enzyme-regulating factors whose mode of action might be elucidated by the study of hormone-enzyme relationships. Nor as yet is anything known as to how a hormone acts in producing an abnormal cellular state.

Until this problem is more fully appreciated, it will be impossible to comprehend the factors involved in inducing an endocrine-dependent cancer to develop spontaneously into an unrestrained growth or autonomous variant. In the particular mouse prostatic carcinoma which I have mentioned, the cancer cell before it metamorphoses from a hormone-dependent into an autonomous lesion undergoes a pronounced cellular differentiation, and by histological examination *it is therefore possible* to predict beforehand the response of this tumour transplant to hormonal modification.

Unfortunately this phenomenon is the exception to the rule. Nevertheless it might provide suitable material when one endeavours to elucidate certain aspects of this fundamental problem. The differences in the behaviour of endocrine and non-endocrine-dependent neoplasms are important when thinking in terms of chemotherapy. It is not possible in other types of

Let me conclude as I began by quoting a pertinent remark by Dr. Jacob Furth. 'Recognition and adjustment of the hormonal disturbance in this disease is in fact cancer prophylaxis.'
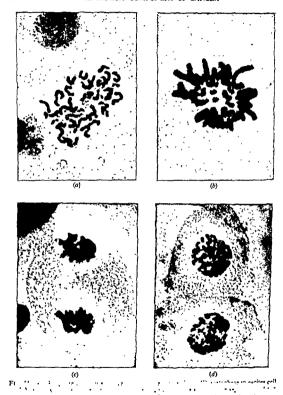
REFERENCES

BIELSCHOWSKY, F. (1954). *Proc. Univ. Otago*, **33**, 16.

CADE, Sir S. (1955). *Brit. med. J.* **1**, 1.

COOK, J. W. and Dopps S'- F. C. (————) M' . ::

COWIE, A. :

CRAMER, '.

ECK, VAN V. and CHANG, C. H. (1955). *Cancer Res.* **15**, 280.

ENGEL, L. L. (1955). Personal communication.

FURTH, JACOB (1955). *Recent progress in Hormone Research*, p. 221. Academic Press, New York.

HADDOW, A., WATKINSON, J. M. and PATTERSON, E. (1944). *Brit. med. J.* ii, 393.

HADFIELD, G. (1956). *Brit. med. J.* **1**, 94.

HORNING, E. S. (1946). *Lancet*, Dec. 7, 829.

HORNING, E. S. and WHITTICK, J. W. (1954). *Brit. J. Cancer*, **8**, 451.

HUGGINS, C. and HODGES, C. V. (1941). *Cancer Res.* **1**, 293.

HUGGINS, C. and MOULDER, P. H. (1945). *Cancer Res.* **5**, 510.

HUGGINS, C. and SCOTT, W. W. (1945). *Ann. Surg.* **122**, 1031.

K————.————. T. . . T'. . ' ' . . . . : ' ;

385.

LOESER, A. (1940). *Brit. med. J.* **1**, 479.

MIRAND, E. A. (1955). Personal communication.

MÜHLBOCK, O. (1954). Congrès international de Gynécologie et d'Obstétrique, Geneva.

NATHANSON, I. T. and ANDERVONT, H. B. (1939). *Proc. Soc. exp. Biol. and Med.* **40**, 421.

NATHANSON, I. T. (1947). *Endocrine Aspects of Cancer*, p. 26, Academic Press, New York.

PEARSON, O. H , WEST, C O. and HOLLANDER, V. P. (1954). *J. Amer. med. Ass.* **158**, 234.

PEARSON, O. H. (1955). *J. Amer. med. Ass.* **159**, 1701.

Data is now accumulating on the effects of hypophysectomy on patients suffering from breast cancer. Some of these reports deal with patients having disseminated carcinoma who have had an adrenalectomy but have suffered a relapse after varying periods of remission. In the last resort a hypophysectomy has been done. In several of these cases a further rapid regression of the carcinoma has been obtained. In others in which reactivation of the growth had occurred, following gonadectomy and adrenalectomy, post-mortem examination has revealed the presence of accessory cortical adrenal rests. These were apparently the cause of the reactivation of the growth. Recurrences of this kind after a remission, in the absence of ovaries and adrenal glands suggests that the anterior pituitary, in attempting to maintain an equilibrium, is capable of stimulating the hypertrophied adrenal rests to secrete oestrogen in sufficient amounts to reactivate the quiescent neoplasm. Pearson contends that there is also evidence suggesting that the reoccurrence of some breast carcinomas is due to a pituitary hormone or hormones acting in addition to oestrogen. Indeed, accessory cortical tissue is not always found in cases which have undergone reactivation. One wonders whether prolactin acting alone or in combination with other pituitary hormones will not eventually be found to play an important role in this complicated story.

Remission of endocrine cancer, whether obtained by a hormone antagonist, or by depression of pituitary function by oestrogens, or by surgical removal of the gonads and adrenals, is only temporary in duration. Sir Stanford Cade (1955) in a recent review of one hundred cases states that remission following adrenalectomy and maintenance on cortisone varies from several months to three years. Huggins of Chicago, who initiated this form of surgical therapy, claims remissions, in some instances up to six years.

There are many gaps in our knowledge and perhaps more concentrated study of some of the anomalies in endocrine carcinogenesis will help in the final solution of this problem. The fact that some types of endocrine cancer can in many instances be fully or partly controlled by an alteration of the hormonal environment of the host is, to say the least, encouraging.

All material for the cytological studies shown in this illustration was prepared by the method and stained with acetic-orcein (see Koller 1953c). (Magnification × 2,600)

THE GENETIC COMPONENT OF CANCER

P. C. KOLLER

INTRODUCTION

THERE are three different approaches to the problem of genetics and cancer. Firstly, analysis can be made of those cellular elements which form the physical basis of heredity, namely the chromosomes and genes, so that their behaviour in normal and malignant cells can be compared. Since the classical work of Hansemann (1890) it is known that in tumour cells the chromosomes display various irregularities the causes of which can be studied together with the possible relationship between the process of malignant transformation and mitotic abnormalities and gene mutation.

Secondly, a survey can be made of the accumulated data obtained in the study of experimental tumours. The analyses of spontaneous and induced tumours in animals, particularly in mice, have shown that the origins of mammary carcinoma, lung adenocarcinoma, and leukaemia are under genetic control. The breeding experiments carried out by many experts using various strains of mice, have yielded much information about the role and the complexity of the hereditary factors involved.

Thirdly, the possibility of a hereditary basis of cancer in man is considered. Pedigrees of families in which several members had cancer, often at the same sites, are on record. Can the high incidence of cancer within a family group be taken as evidence for a genetic component in the origin of human cancer?

In this chapter these three aspects are considered and an attempt is made to correlate them for they all converge on the problem of the relationship between genetics and cancer.

BEHAVIOUR OF CHROMOSOMES AND GENES DURING THE DEVELOPMENT OF CANCER

Introduction

Cancer (neoplasm) is a malignant growth which is produced by an excessive multiplication of tissue cells. The most important and essential feature of cell-multiplication or mitosis is the division and distribution of the nuclear content, that is the chromosomes, to the two daughter cells (Fig. 44). As the chromosomes are the carriers of hereditary determinants or genes, mitosis constitutes the central phenomenon in organic reproduction, genetic continuity and heredity (Wilson, 1925).

The transformation of a normal cell into a cancer cell is a permanent, irreversible event and the change is due to a breakdown in the genetic control of cell division. Though this can be initiated by agents of very diverse nature, the results of the transformation are similar, that is an uncontrolled cell multiplication. The following

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